Original Article

Antibacterial, anti-inflammatory, and anti-osteoclastogenesis roles of allicin in periodontitis

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Abstract: Periodontitis is an infectious inflammatory immune disease. Allicin, because of its antibacterial, anti-inflammatory and immunomodulation properties, appears to be a promising drug for treating periodontitis. In this study, the antimicrobial activity of allicin against Porphyromonas gingivalis (P. gingivalis) and its effects on inflammatory cytokine expression in experimental periodontitis and osteoclast activity in vitro were investigated. Allicin inhibited P. gingivalis growth, with a minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 4 mg/l and 8 mg/l, respectively. Allicin showed inhibitory effects on P. gingivalis biofilm formation, with a minimum biofilm inhibition concentration (MBIC) of 4 mg/l and 16 mg/l, respectively, and affected the viability of 1-day-old biofilms with a minimum biofilm reduction concentration (MBRC) of 32 mg/l. Confocal laser scanning microscopy (CSLM) revealed that treatment with 32 mg/l allicin resulted in a lower bacteria number, fewer live bacteria, and a thinner biofilm. In gingivomucosal tissues around first right molar teeth undergoing ligature-induced periodontitis, allicin substantially inhibited periodontal destructive cytokines, including TNF-α, IL-1β, IL-6, INF-γ, and IL-12, but promoted protective cytokines such as IL-4 and IL-10. By affecting the cytokine framework and the secretion of M-CSF and RNAKL from osteoblasts, allicin suppressed osteoclastogenesis by modulating the RANK-RANKL system, as demonstrated by a decrease in NF-κB p65 levels. These results suggested that allicin is a potential natural product for treating periodontitis.

Keywords: Periodontitis, Porphyromonas gingivalis, allicin, growth, biofilm, osteoclastic activity

Introduction

Periodontitis is an inflammatory disorder that continues to be a major oral health problem worldwide [1]. It is caused by the infection with Gram-negative organisms, particularly Porphyromonas gingivalis (P. gingivalis), which is one of the most essential factors in periodontitis. The virulence factors produced by P. gingivalis can cause abnormal host defences [1, 2]. Activated immune cells secrete an abundance of inflammatory cytokines, thereby triggering an aberrant host immune reaction and eventually cause progressive periodontal tissue damage [3]. Among these inflammatory cytokines, TNF-α, INF-γ, IL-1, IL-4, IL-6 and IL-12 have been shown to be involved in the pathogenesis of periodontitis [3]. In addition to the control of invasive pathogens at the cost of periodontal tissue damage via induction of a cellular immune reaction, these factors could also activate the receptor activator of nuclear factor-κB (NF-κB) (RANK)-RANKL system, the stimulation of which could induce elevated resorption of alveolar bone [4].

The treatment for periodontitis generally includes mechanical debridement, periodontal surgery and antibacterial agents [5]. Mechanical debridement is performed to remove dental plaque, but the host’s unique immune reaction in periodontitis is ignored [3]. Periodontal surgery is not the only non-ideal therapeutic strategy for advanced periodontitis, but it also presents a financial consideration in developing countries. Regarding antibiotics, undesirable side effects, such as drug resistance, vomiting, and tooth staining, limit their clinical application [6]. Hence, alternative products from natural plants used as traditional medicines are considered as alternatives. Allicin may be a promising anti-microorganism agent.
Allicin for periodontitis treatment

Allicin, a garlic extract that is known for the production of organosulfur compounds, exhibits varying biological and pharmacological functions. The chemical compound not only acts against microbial infections but also shows cardioprotective, anti-cancerogenic, and anti-inflammatory activity [7-10]. Although no studies have described the role of allicin in treating periodontitis, several researchers have shown that allicin inhibits the adhesion, growth and biofilm formation of bacteria, including Escherichia, Salmonella, Staphylococcus, Streptococcus, Klebsiella, Proteus, Clostridium, and Mycobacterium [11]. In addition, by regulating systemic inflammatory cytokines such as TNF-α, INF-γ, IL-1, IL-4, IL-6 and IL-12, allicin could control the body’s inflammatory response [4, 12-16]. Decreased expression of these pro-inflammatory factors is helpful in relieving NF-κB activation and osteoclastic activity [4]. In addition, with mechanisms including modulation of cytokine secretion as described above, immunoglobulin production, and macrophage activation, allicin can regulate the stability of the immune system by stimulating macrophages, lymphocytes, natural killer (NK) cells, and other types of immune cells [4, 12-16].

Interestingly, all biological effects shown by allicin are beneficial for periodontitis treatment. It is thus reasonable to hypothesize that allicin may be a potential natural product for the treatment of periodontitis. In this study, the antimicrobial activity of allicin against the growth and biofilm formation of P. gingivalis, inflammatory cytokine levels in experimental periodontitis after allicin washing, and in vitro osteoclastogenesis after allicin treatment were examined to demonstrate that allicin is a promising drug for periodontitis treatment.

Materials and methods

Chemicals, bacterial strain, growth medium and growth conditions

Allicin was supplied by Shanghai Guangrui Bioscience Inc., Shanghai, China. All other reagents, unless otherwise stated, were purchased from Sigma (St. Louis, MO, USA). P. gingivalis (ATCC33277) was obtained from the State Key Laboratory of Oral Disease (Chengdu, Sichuan, China). TSB-yeast extract medium for culturing P. gingivalis was supplemented with 0.02 μg/ml menadione, 0.05% cysteine hydrochloride, 0.02% potassium nitrate, and 5 μg/ml hemin. P. gingivalis subcultures were incubated in an anaerobic chamber containing 10% H₂, 5% CO₂ and 85% N₂ at 37°C.

Minimal inhibitory (MIC) and minimal bactericidal concentration (MBC) determination

The assays were performed by a modification of that described by Cutler and Wilson [17]. For MIC measurement, two-fold serial dilutions of allicin, ranging from 0.5 mg/l to 16 mg/l, were prepared at volumes of 100 μl/well in 96-well plates. Then, a 20-μl bacterial cell suspension was added to each well at a final concentration of 1 × 10⁶ colony-forming units (CFU)/ml. After the cells were subcultured for 1 d, the MIC was defined as the lowest concentration at which no visible bacterial growth against a black background was observed. Similarly, to determine the MBC, we prepared aliquots of bacterial cell suspension in the 96-well plates with an allicin concentration greater than the MIC. The MBC was defined as the lowest concentration at which no surviving cells were detected on the agar.

Time-kill curve

A time-kill kinetic curve was obtained as previously described using the microdilution method [17, 18]. The allicin concentrations used in the assay corresponded to 1/2 the MIC, MIC and MBC. At specified time intervals (0 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 18 h and 24 h), samples were serially diluted, re-plated onto new medium and counted with a colony counter (Synbiosis Acolyte 7510; Microbiology International, MD, USA). The curve was then drawn by plotting log₁₀ CFU/ml versus time (h).

Biofilm susceptibility assay

Aliquots of bacterial suspension, as described above, were subcultured in an anaerobic environment at 37°C for 1 d. After treatment with allicin concentrations ranging from 0.5 mg/l to 32 mg/l, samples were washed with PBS, fixed with methanol for 15 min, stained with 0.1% (w/v) crystal violet for 5 min and rinsed with ddH₂O until no colour was visible in the control well. Aliquots of 95% ethanol were added for 30 min to dissolve the crystals. OD values (A₅90) were measured by a Microplate Reader (Bio-Rad, Hercules, CA, USA). The minimum biofilm inhibition (MBICₕ₀/MBICₚ₀) was defined as the lowest concentration at which 50% or 90% of
biofilm formation was inhibited. To determine the minimum biofilm reduction concentration (MBRC<sub>50</sub>), culture medium with high allicin concentrations was added to 1-day-old biofilm and incubated for an additional 24 h. By assessing OD values at A<sub>590</sub>, the MBRC<sub>50</sub> was calculated as the lowest concentration that could reduce the formation of biofilm by ≥ 50%.

Confocal laser scanning microscopy (CLSM)
A one-day-old biofilm was formed on Lab-Tek<sup>®</sup> Chamber Permanox<sup>™</sup> slides (Nagle Nunc International, NY, USA). Culture medium containing 32 mg/l allicin (MBRC<sub>50</sub>) was added to the wells and incubated for one additional day. Slides were stained for 15-20 min in a dark room with the LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> Bacterial Viability Kit (Molecular Probes Inc., OR, USA). The live/dead cells were viewed under a Leica TCS SP2 microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany).

Periodontitis model establishment and allicin treatment
Animal care and experiments were performed in accordance with guidelines issued by the Institutional Animal Care and Use Committee, Zhejiang University, Hangzhou, China. Animals were randomly divided into four groups: sham (n=8), periodontitis (n=9), periodontitis + allicin treatment at the MIC (n=9), and periodontitis + allicin treatment at the MBC (n=9). To establish a periodontitis model, Sprague-Dawley rats (374 ± 45 g body weight) were anesthetized with 4% isoflurane vapour, and a sterile 28-gauge wire ligature was tied around the cervical portion of the right first molar [19]. The experimental rats were monitored daily to ensure the presence of the ligature. After ligature induced-periodontitis models were established, for allicin treating, animals were anesthetized with 4% isoflurane vapour, then injected with 2 ml PBS with or without allicin (4 mg/l or 8 mg/l) on the palatal gingiva of the first molars, as previously described [20]. At seven days, gingivomucosal tissues surrounding the molar teeth were harvested for further experiments, including cytokine analysis and western blotting.

Gingivomucosal cytokine analysis
The digestion solution for the collected gingivomucosal tissues included 50 μg/ml gentamicin, 500 U/ml collagenase, 100 μg/ml zwittergent-12, 10 mM HEPES buffer, and 100 μg/ml bovine serum albumin in RPMI 1640 [21]. The samples were minced, weighed, digested, sonicated, and centrifuged. The TNF-α, IL-1β, IL-4, IL-6, IL-10, INF-γ and IL-12 levels in supernatants were determined using a commercial colorimetric kit (R&D Systems Inc., USA). All cytokine concentrations are expressed as pg/mg tissue.

Western blotting
The protein samples collected from gingivomucosal tissues were solubilized in lysis buffer, as previously described [22]. To measure NF-κB p65 levels from nuclear fraction of the harvested samples, we separated the samples on 8% SDS-PAGE gels, transferred them onto polyvinylidene difluoride membranes, blocked them in 5% non-fat milk for 1 h and incubated them with specific primary antibodies overnight at 4°C. After the samples were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature, protein bands were visualized with
enhanced chemiluminescence reagents using a Pico kit. The intensities of bands were assessed by Scion Image Beta 4.0.2 software (Scion Corporation, MD, USA).

**Cell culture, allicin treatment and in vitro osteoclastogenesis assay**

**Osteoblast and osteoclast precursor isolation and co-culture:** Osteoblasts and bone marrow cells were isolated from the tibias and femurs of the experimental Sprague-Dawley rats, as our previous study published in “Bone” described [23]. The adherent bone marrow macrophages (BMMs) were chosen as osteoclast precursors. BMMs (2 × 10^4, passage 2-4) were cultured in 24-well plates with α-MEM medium + 10% FBS. Transwell chambers (pore diameter: 3 μm; Corning) that were cultivated with 1 × 10^5 osteoblasts were placed into the 24-well plates. The co-culture systems were then treated with 4 or 8 mg/l allicin for 4 days.

**Enzyme-linked immunosorbent assay (ELISA):** The supernatants were collected from co-cultured medium on day 4. RANKL and M-CSF levels were quantified using ELISA kits (R&D Systems Inc., USA). The cytokine concentrations are presented as the amount (pg) of each cytokine per ml of supernatant.

**Tartrate resistant acid phosphatase (TRAP) staining:** Cells were fixed in a solution of 4% formaldehyde for 30 sec, incubated in TRAP staining solution for 1 h at 37°C, rinsed in deionized water, air-dried and evaluated microscopically. Eight fields under a × 4 objective were randomly selected for each well. The numbers of TRAP+ multinucleate (at least three nuclei) cells (MNCs) were counted and recorded as the number of osteoclasts.

**Statistical analysis**

All data were collected from triplicate experiments reproduced at least three times. Data
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Results

Antibacterial activity of allicin against P. gingivalis in planktonic culture

Allicin showed an inhibitory effect on P. gingivalis with a MIC of 4 mg/l. An allicin concentration of 8 mg/l could completely kill P. gingivalis in planktonic culture (Table 1). To dynamically assess the antimicrobial activity of allicin, time-kill kinetic studies were further performed. As presented in Figure 1, the cell-killing effects exhibited by allicin on P. gingivalis were time- and concentration-dependent. When the drug level reached 8 mg/l (MBC), the time-kill curve showed a trend similar to that observed with 0.05% chlorhexidine (Figure 1).

Inhibitory/destructive effects of allicin on P. gingivalis biofilm

Allicin could inhibit biofilm formation by P. gingivalis, with an MBIC_{50} of 4 mg/l and an MBIC_{90} of 16 mg/l (Table 1). As the drug levels increased, 32 mg/l of allicin (MBRC_{50}) could suppress the viability of previously formed P. gingivalis biofilms.

To further ensure the effects of allicin on previously-formed P. gingivalis biofilms, we conduct-

The biofilm thicknesses in each group were measured by CLSM. In the control group, the thickness was 43.1 ± 5.3 μm. However, the biofilm was thinner in the allicin-treated group (27.4 ± 4.1 μm; Figure 3B; *P<0.05). The results suggested that allicin not only killed P. gingivalis within the biofilm but also disrupted the environment of P. gingivalis by disintegrating the biofilm.

Allicin-induced modulation of inflammatory cytokine expression and NF-κB p65 levels in gingivomucosal tissues around first right molar teeth undergoing ligature-induced periodontitis

As shown in Table 2, destructive cytokines, including TNF-α, IL-1β, INF-γ, IL-6 and IL-12, exhibited dramatically elevated levels in experimental rat periodontitis on day 4 (*P<0.05). Although allicin was unable to revert these cytokine levels to a normal status, they were much lower in the allicin-treated group (P<0.05). It is worth noting that allicin not only significantly inhibited the inflammatory cytokine levels but also showed the ability to promote the secretion of IL-4 and IL-10 (*P<0.05), two cytokines that protect against periodontitis. These data thus suggested that allicin could change the cytokine framework in periodontitis from a “destructive” to a “protective” pattern. As the drug concentration increased (8 mg/l, MBC), this favourable effect became more apparent (*P<0.05).

To determine whether allicin could relieve the abnormal osteoclastic activity that occurs in

Figure 3. The ratios of live bacteria and the average thicknesses of P. gingivalis biofilms treated with allicin. A. The percent of live bacteria was significantly decreased after allicin treatment. B. The thickness of biofilms was dramatically reduced after allicin treatment. Data are presented as the mean ± SD (n>3). *P<0.05 vs Allicin-untreated controls.
periodontitis, we detected the level of NF-κB p65, a key factor in the RANK-RANKL system. Western blotting showed that allicin inhibited the expression of NF-κB p65 in a concentration-dependent manner (Figure 4; *P<0.05). The reduction of inflammatory cytokine levels by allicin may cause inhibition of osteoclastic activity.

**Discussion**

Periodontitis is not only a chronic infectious disease but also an inflammatory immune disorder. Due to increased bacterial resistance to antibiotics and an increasing focus on the unique immune response in periodontitis, considerable concern exists regarding the development of other antibacterial agents. These antibacterial agents should not only have few side effects but should also be able to control the abnormal immune reaction that occurs in periodontitis. The benefits of garlic on human health have been previously demonstrated. Allicin, a garlic extract, has been shown to have antibacterial and anti-inflammatory effects, which makes it a promising agent for the treatment of periodontitis. In this study, we investigated the inhibitory effects of allicin on periodontitis by assessing the expression levels of key inflammatory cytokines and the RANK-RANKL system. Our results indicate that allicin significantly reduces the levels of inflammatory cytokines and inhibits the expression of NF-κB p65, suggesting its potential as a novel therapeutic agent for periodontitis.
have an antibacterial property [11]. It also has been proposed as a promising natural product for maintaining the stability of the immune system [4, 12-16]. Hence, local application of allicin may be a helpful for the treatment of periodontitis.

P. gingivalis is regarded as one of the most important microorganisms in the occurrence and disease process of periodontitis [3]. Allicin not only affects the survival of P. gingivalis, but also inhibits and kills other bacteria, including Escherichia, Salmonella, Streptococcus, Proteus, Clostridium, Mycobacterium and Helicobacter species [11]. Allicin thus has a wide spectrum of antibacterial activity. Periodontitis represents the infectious outcome of many Gram-negative bacteria. The favourable actions of allicin on some of these Gram-negative species are helpful in treating periodontitis, and these bacteria species should be clarified in further investigations.

Bacteria that generally reside in the mouth form dental plaques comprising a highly organized microorganism community [24]. Bacteria in these plaques are less susceptible to antimicrobial agents. To inhibit new biofilms or destroy previously-formed biofilms is thus of great importance for anti-periodontitis drugs. In this study, we showed that allicin exhibited inhibitory effects on P. gingivalis biofilm formation and 1-day-old biofilm with a MBIC_{50} of 4 mg/l, MBIC_{90} of 16 mg/l, and MBRC_{50} of 32 mg/l. CLSM indicated that the action of allicin on biofilm is a result of bacterial growth inhibition and killing effects because the biofilm thickness in the allicin-treated group was significantly decreased, and the number of red-stained cells (dead bacteria) was dramatically increased. In contrast, damage to the organized microstructure in P. gingivalis biofilm also serves as positive feedback for allicin action because the disintegrated biofilm is more vulnerable to antimicrobial agents.

In addition to controlling bacterial infection, immunomodulation and inflammatory regulation need to investigated for periodontitis treatment in the future. This could be examined by dynamic regulation of proinflammatory cytokines such as TNF-α, IL-1β, INF-γ, IL-6 and IL-12. TNF-α, which is highly expressed in gingival crevicular fluid, is positively associated with periodontal tissue damage [25-27]. In addition to directly promoting RANKL expression, TNF-α acts as an important chemotactic cytokine and inducer and could stimulate the production of other pro-inflammatory immune cytokines, including IL-1β, IL-6 and INF-γ [25-27]. INF-γ exerts positive feedback and could further promote the expression of TNF-α [28]. All of these cytokines and chemokines have been shown to be highly expressed in periodontitis, causing rapid cell migration, elevated osteoclastogenesis by promoting RANKL expression, and increased bone absorption [3, 27].

These chemokines and cytokines not only trigger an acute inflammatory response, but some are also involved in periodontitis-related abnormal immune reactions. IL-6 is associated with B-cell differentiation and antibody production [29]. The autoantibodies produced by B cells, including collagen, vimentin, and actin, serve as immunogens and will result in aggressive periodontitis [30]. The deletion of B-cells is helpful in preventing bone loss in mice that suffer from P. gingivalis infection [31, 32]. In addi-
tion, INF-γ and IL-12 are mainly associated with T-cell activation [28, 33]. Th1 cell stimulation not only induces an abnormal adaptive immune response and progressive periodontal damage but is also a stimulus for RANKL accumulation and alveolar bone loss [33].

Interestingly, by inhibiting these destructive cytokines, allicin exhibits phytotherapy for periodontitis treatment. Moreover, allicin can increase levels of IL-4 and IL-10, which are two cytokines that protect against periodontitis [34-37]. The upregulation of IL-10 may cause downregulation of the proinflammatory cytokines TNF-α, IL-1β, INF-γ, and IL-6 and increase the body’s resistance to P. gingivalis, preventing osteoclastogenesis via inhibition of the RANK-RANKL system and reducing the severity of periodontitis [38, 39]. IL-4 also presents anti-inflammatory properties due to its capacity to inhibit INF-γ expression and Th1 cell stimulation [36, 37]. Hence, by changing the cytokine framework from a destructive pattern into a protective mode, allicin could relieve tissue damage during the periodontitis.

Alteration of the cytokine framework is beneficial for controlling osteoclastic activity. Osteoclastogenesis requires cell-cell contacts with osteoblasts/stromal cells and secreted soluble cytokines including RANKL and M-CSF [4]. Our in vitro data showed that allicin indirectly affected osteoclasts by influencing the production of RANKL and M-CSF. Therefore, osteoblasts or other stromal cells may also be responsible for allicin-induced inhibition of osteoclastic activity.

Thus, daily intake of allicin or mouth washing may to some extent be beneficial in treating or preventing periodontitis.

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Disclosure of conflict of interest

None.

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