Original article

Effects of S-PRG Eluate on Bacterial Activity Related to Periodontitis and Oral Malodor

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Key words : S-PRG, periodontitis, oral malodor, volatile sulfur compounds, Porphyromonas gingivalis

Abstract : Periodontitis is mainly caused by some gram-negative bacteria, and oral malodor is associated with volatile sulfur compounds (VSCs) produced by these bacteria. Our long-term objective is to identify/develop new products with the ability to reduce periodontitis and oral malodor as an alternative to the use of antibiotics or antiseptics. In this study, we hypothesized that surface pre-reacted glass ionomers (S-PRG) release ions that inhibit bacterial activity associated with periodontitis and oral malodor. More specifically, we investigated the effects of S-PRG on proteolytic activity, VSC production, and bacterial interactions in *Porphyromonas gingivalis*. Twenty percent of S-PRG eluate significantly reduced the growth of *P. gingivalis* ($p < 0.05$). Twenty percent of S-PRG eluate also reduced the production of hydrogen sulfide and methyl mercaptan about 34.1% and 42.0% respectively, although the difference was not significant. The S-PRG eluate reduced the proteolytic activity of *P. gingivalis*, and the actual ion, which inhibited the protease activity was found to be boron ion. *Tannerella forsythia* extracts significantly promoted the growth of *P. gingivalis*, but this effect was abolished in the presence of S-PRG eluate. Our findings indicate that ions released from widely-used dental materials containing S-PRG may have a beneficial effect against periodontitis and oral malodor.

1. Introduction

After dental caries and periodontitis, oral malodor, also known as bad breath and halitosis, is estimated to be the third most frequent complaint of patients seeking dental care[1]. Approximately 90% of oral malodor are associated with oral conditions such as periodontitis, tongue coating, dry mouth, and poorly fitting restorations; others are caused by systemic diseases, including otolaryngological infections, gastrointestinal disorders, hepatic diseases, and diabetes[2–4]. The major compounds that contribute to oral malodor are volatile sulfur compounds (VSCs) such as hydrogen sulfide (H_2S), methyl mercaptan

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(CH_3SH), and dimethyl sulfide (CH_3SCH_3)[5,6]. These malodorous compounds are produced in the oral cavity during the process of protein metabolism by oral bacteria. VSCs are mainly produced by gram-negative bacteria, which are also associated with periodontitis. The red complex bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) have been strongly associated with the progression and severity of periodontitis[7]. More specifically, *P. gingivalis* strongly produces hydrogen sulfide and methyl mercaptan after proteolytic digestion of intra-oral proteins[4].

The human oral cavity contains more than 700 bacterial species that interact with each other and with host tissues; numerous gram-negative bacteria found in periodontitis sites and tongue coating may be related to malodor production[8,9]. Moreover, recent studies have suggested that gram-positive bacteria enhance the production of VSCs by gram-negative bacteria[10-12]. Therefore, it is important to control both gram-positive and gram-negative bacteria in the oral cavity to reduce oral malodor.

Antibiotics and antiseptics are effective in reducing oral malodor, but due to the side-effects and possible emergence of drug-resistant bacteria, with the subsequent possibility of microbial substitution, a long-term use of those is not advisable. Nowadays, there is an interest in research aimed to identify/develop novel strategies to control bacteria-related halitosis. Our laboratory previously reported that tablets containing probiotic bacteria may be effective in controlling the oral flora and reducing the production of VSCs[13]. We are currently investigating hinokitiol and catechins, which are known to control oral flora without exhibiting negative impacts on human health[14,15]. Recently, we initiated research on a novel strategy based on the potential of dental materials to reduce oral malodor.

A multi-ion releasing dental material, surface pre-reacted glass ionomer (S-PRG), is widely used for dental treatment. S-PRG filler particles are formed by an acid-base reaction between fluoroaluminosilicate glass and polyacrylic acid[16] and are capable of fluoride (F^-) release and recharge[17,18]. In addition to F^-, S-PRG is also known to release several ions including aluminum (Al^{3+}), strontium (Sr^{2+}), boron (BO_3^{3-}) and so on[19,20], which may affect several activities of the periodontopathic bacteria.

Recently, we reported that oral malodor was reduced by rinsing with S-PRG eluate[21]; however, the mechanism by which this occurs remains unclear. One of the possible mechanisms of this oral malodor reduction is the inhibitory effect of S-PRG eluate on *P. gingivalis*. In this study, therefore, we investigated the effects of S-PRG eluate on VSC production, proteolytic activity and bacterial interactions in *P. gingivalis*.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

P. gingivalis ATCC 33277 was maintained on CDC anaerobic blood agar (Becton Dickinson, Cockeysville, MD, USA) at 37℃ in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2), and inoculated into tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, USA) supplemented with hemin (5 μg/mL) and menadione (1 μg/mL). *T. forsythia* ATCC 43037 was grown on CDC anaerobic blood agar at 37℃ together with *Fusobacterium nucleatum* ATCC 23726 to accelerate its growth, and transferred to Brain Heart Infusion broth containing 0.001% N-acetyl-muramic acid (Sigma Chemical Co., St. Louis, MO, USA) and 5% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA).

2.2. Preparation of sonicated extracts of *P. gingivalis* and *T. forsythia* cells

P. gingivalis and *T. forsythia* cells at the late logarithmic stage in 500 mL TSB were harvested by centrifugation and washed with phosphate-buffered saline (PBS). Bacterial cell pellets were suspended in 5 mL of PBS, and the cells were disrupted by sonication on ice[22]. Intact cells were removed by centrifugation and the supernatant was defined as a sonicated extract (SE).

2.3. Preparation of S-PRG eluate

S-PRG eluate was prepared by the method of Fujimoto et al[19]. and the condition that produces a

high concentration of each ion was applied. Briefly, S-PRG filler (Shofu Inc. Kyoto, Japan) was mixed with an equal amount of distilled water and shaken gently at room temperature for 24 h. Filler material was removed by filtration. The ion solution was centrifuged to remove any residual insoluble material, and the clear supernatant collected was used as the S-PRG eluate. Elemental analysis of ions released from the S-PRG filler was performed using inductively coupled plasma atomic emission spectroscopy (ICP-AES; ICPS-8000, Shimadzu Co., Kyoto, Japan). Analysis was conducted after preparing calibration curves corresponding to each element (standard solution concentration; Sr: 0, 5, 20, and 50 ppm; B: 0, 10, 50, and 100 ppm; Al: 0, 0.5, 5, and 10 ppm). Concentration of F was also determined using a fluoride ion electrode method after preparing its calibration curves (standard solution concentration: 0.1, 1, 5, and 10 ppm). A fluoride electrode (Model 9609BN, Orion Research Inc., MA, USA) connected to a pH/ion meter (720A, Orion Research Inc.) was used to measure the F concentration of each solution. Each test solution was diluted to obtain a fluoride ion level of less than 5 ppm, and 0.5 mL of an ionic strength adjuster (TISAB III, Orion Research Inc.) was added to 5 mL of each diluted solution to measure F ion concentration. For each ion under investigation, the amount released into the solution was expressed in ppm, as well as a cumulative amount per gram of S-PRG filler weight (mg/g).

2.4. Measurement of growth and VSCs produced by *P. gingivalis*

P. gingivalis was cultured with TSB supplemented with hemin and menadione. To examine the effect of S-PRG eluate, TSB was replaced with distilled water or S-PRG eluate. After culturing in a 15ml-plastic tube for 24 h, the optical density of culture medium at 600nm was measured, and the head space air was collected with a gas-tight syringe and 1 ml of the air was applied to Oral Chroma® (Nissha FIS, Inc. Osaka, Japan).

2.5. Protease activity of *P. gingivalis* SE

N_{α}-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) was purchased from Sigma Aldrich (St. Louis, MS, USA). BAPNA is a synthetic substrate, which develops a yellow color when arginine residues are cleaved by trypsin-like proteases. An enzyme assay was performed according to the method of Potempa et al[23]. Briefly, *P. gingivalis* SE was added to an aqueous reaction mixture containing 1 mM BAPNA, 0.2 M Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $CaCl_2$, and 10 mM cysteine. For the control, *P. gingivalis* SE was omitted from the reaction mixture. The background color was calculated by measuring the optical density of *P. gingivalis* SE. To analyze the effect of S-PRG eluate, distilled water in the reaction mixture was replaced with S-PRG solution. The enzyme assay mixture was incubated at 37℃ for 30 min and the absorbance at 405 nm was measured to determine colored metabolites (as a measure of protease activity). To identify the ions that actually suppressed the proteolytic activity, each ion presenting in the S-PRG eluate was added to the reaction mixture. To prepare the ion solution, standard solutions of fluoride, aluminum, strontium, and boron (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were diluted with distilled water.

2.6. Growth promotion of *P. gingivalis* by *T. forsythia* SE

Growth promotion was examined by using the modified method of Takahashi and Sato[24]. TSB was diluted to 40% of the original concentration with PBS and supplemented with hemin and menadione (diluted TSB). Then, 100 μl of *P. gingivalis* suspension was inoculated into 5 ml of diluted TSB, with or without *T. forsythia* SE. Diluted TSB supplemented with *T. forsythia* SE was incubated at the same time and served as a blank. Bacterial growth was measured as optical densities at 600 nm. To analyze the effect of S-PRG eluate, 10 or 20% of S-PRG eluate was added to the diluted TSB.

2.7. Statistical analysis

All experiments were performed at least three times for reproducibility. Statistical analyses were performed using SPSS® version 25 (IBM Corp, Armonk, NY, USA). The difference of production of hydrogen sulfide and methyl mercaptan between each S-PRG eluate were evaluated by Mann-Whitney analysis. Kruskal-Wallis test and post-hoc Mann-Whitney analysis corrected with Scheffe's test were performed to detect the difference of protease activity and bacterial growth. p values of <0.05 were considered statistically significant.

3. Results

3.1. Nature of S-PRG eluate

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) and the fluoride ion electrode method allowed to determine the ion concentration of S-PRG eluate (Table 1). The pH of the eluate was 7.8 and all the experiments were performed with the same batch of S-PRG eluate. No pH change or precipitation occurred when the eluate was added to the reaction mixtures.

3.2. Effect of S-PRG eluate on *P. gingivalis* growth and VSC production

Twenty percent of S-PRG eluate significantly reduced the growth of *P. gingivalis* (Figure 1, $p<0.05$). The headspace air of the *P. gingivalis* culture tube was analyzed for VSCs with Oral Chroma®. When 20% of S-PRG eluate was added to TSB, the production of hydrogen sulfide and methyl mercaptan was reduced (34.1% and 42.0%, respectively), although there was no significant difference (Figure 2).

3.3. Effect of various ions on protease activity of *P. gingivalis*

The ability of S-PRG to inhibit the protease activity of *P. gingivalis* has been previously demonstrated, but it is not yet known which ions cause this effect. We examined the effect of several ions that are constituents of S-PRG eluate on the protease activity of *P. gingivalis* using the Arg-

Table 1 Concentration of ions in the S-PRG eluate. Concentrations of ions present in the S-PRG eluate were expressed as ppm and mM.

	BO_3^{3-}	F^-	Al^{3+}	Sr^{2+}
ppm	1368.4	127.5	18.4	167.1
mM	126.59	6.71	0.68	1.91

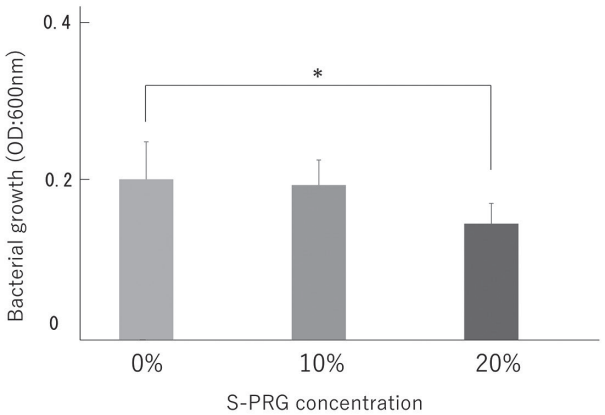

Figure 1 Effect of S-PRG eluate on the growth of *P. gingivalis*. *P. gingivalis* was cultured in TSB; 10% and 20% of distilled water in the reaction mixture was substituted with S-PRG eluate, and the optical density of the culture was measured after 40 h of incubation. *: Significant difference compared to control (0% S-PRG) (*$p<0.05$).

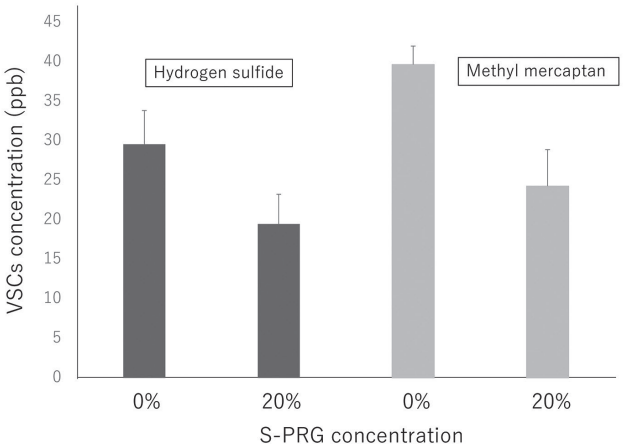

Figure 2 Effect of S-PRG eluate on volatile sulfur compound production by *P. gingivalis*. *P. gingivalis* was cultured for 24 h in the presence and absence of S-PRG eluate. Twenty percent of distilled water in the reaction mixture was substituted with S-PRG eluate, and the headspace air of the culture tube was collected and analyzed for VSCs with an Oral Chroma® apparatus.

gingipain chromogenic substrate. Boron and aluminum ions inhibited the protease activity in a dose-dependent manner (Figure 3A). However, since the actual ion concentrations in the S-PRG eluate are different (Table 1), we added ions at their corresponding concentrations. Boron ions were found to have the strongest inhibitory effect and the protease activity was reduced to 67% of the control (Figure 3B, $p < 0.05$).

3.4. Effect of S-PRG on growth promotion of *P. gingivalis* by *T. forsythia* SE

S-PRG eluate inhibited the growth of *P. gingivalis* in diluted TSB in a dose-dependent manner, but there was no significant difference (Figure 4A). *T. forsythia* SE promoted the growth of *P. gingivalis* in diluted TSB, and 720 μg/ml of *T. forsythia* SE promoted the growth of *P. gingivalis* (Figure 4B). S-PRG eluate eliminated this growth- promoting effect (Figure 4C). The bacterial growth after a 40-h incubation is summarized in Figure 4D. Seven-hundred and twenty μg/ml of *T. forsythia* SE significantly promoted the growth of *P. gingivalis* ($p < 0.05$), and addition of 20% S-PRG eluate eliminated this growth-promoting effect ($p < 0.05$).

4. Discussion

S-PRG fillers are widely used in many fields of dentistry, such as composite resins, cements, dentures, and sealants [25], mainly because of their ability to recharge and release fluoride ions, thus promoting dentin remineralization.

The anti-bacterial activities of S-PRG eluate have been investigated by many researchers, and were recently reviewed by Yoneda et al.[26]. In addition, Imazato et al. summarized the precise mechanism of these anti-bacterial activities[27]. Although S-PRG does not have a strong bactericidal activity, it has growth-suppressing and anti-biofilm effects[21]. These properties are advantageous for caries prevention and the control of periodontal disease[28].

Periodontopathic bacteria are associated with oral malodor, that represents a problem for a large proportion of the population worldwide[29]. Consequently, controlling periodontopathic bacteria with S-PRG may be an interesting strategy to reduce oral malodor.

P. gingivalis is known to produce VSCs by degrading endogenous and exogenous proteins in the oral cavity. Suzuki et al reported that rinsing with S-PRG eluate significantly reduced the oral malodor[21]. Therefore, we examined the effect of S-PRG eluate on VSC production by *P. gingivalis*. There are several mechanisms by which VSCs production can be reduced. One method is to reduce the growth of *P. gingivalis* by S-PRG eluate. In our experiment using liquid medium, S-PRG eluate attenuated the growth rate, and the result is consistent with a previous study [21]. The VSCs concentration produced by *P. gingivalis* was reduced when S-PRG eluate was added to the culture medium, but the reduction of VSCs was not significant in this experiment. It may be because of technical limitation. We can measure only one sample at one time with an Oral Chroma® apparatus, and high amount of VSCs in the headspace of tube may have vanished during the long-time experiment.

P. gingivalis is known to have strong protease activity, and the proteolytic activity is mainly associated with gingipains. Proteases degrade sulfur-containing amino acids and VSCs are produced[30]. Therefore, gingipains are considered to be a potential target to maintain periodontal health and control oral malodor[31]. S-PRG eluate has been previously reported to exert a protease-inhibitory activity[32], but the precise mechanism by which this occurs is not clear.

Some ions in S-PRG eluate seems to possess a protease-inhibiting effect, but it is not yet clear which ion is mostly responsible. Therefore, we investigated four ions (B, F, Al, Sr) that are possible contenders as protease inhibitors of *P. gingivalis*. Among the four ions, boron and aluminum ions exhibited a protease-inhibitory effect in a dose-dependent manner (Figure 2A). Considering the actual concentration in S-PRG eluate (Table 1), aluminum ions did not seem to affect the protease activity under experimental conditions. When the same concentration in S-PRG eluate was applied to the reaction mixture, boron showed the

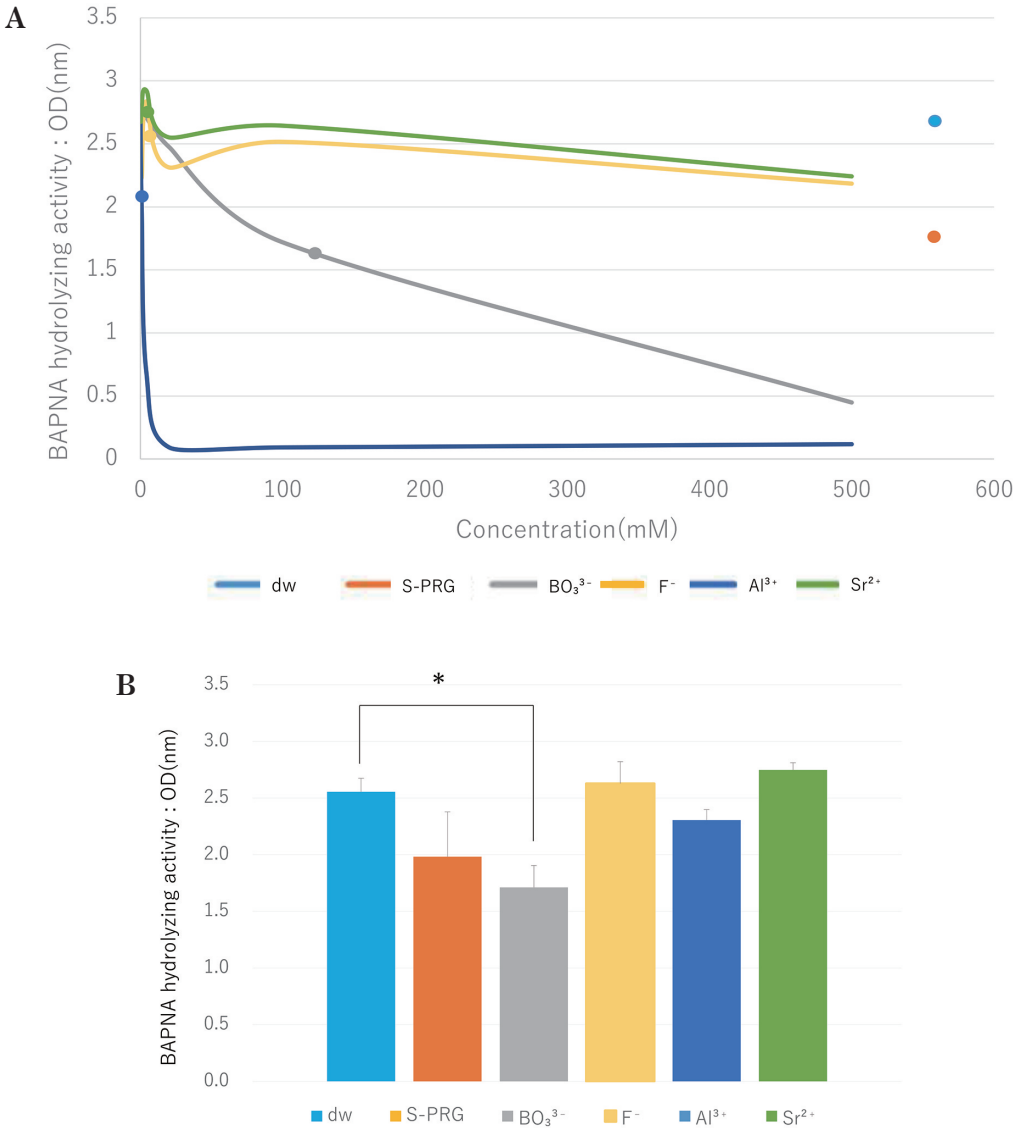

Figure 3

A. Effect of ions on the BAPNA-hydrolyzing activity of *P. gingivalis*. 20% of S-PRG eluate or different concentrations of four ions (BO_3^{3-}, F^-, Al^{3+}, Sr^{2+}) were added to the reaction mixture containing BAPNA and *P. gingivalis* extract. After incubation at 37℃ for 30 min, the absorbance was measured at 405 nm. Light blue dot indicates the OD when no S-PRG eluate was added in the reaction mixture . Orange dot indicates the OD when distilled water was replaced with S-PRG eluate.

B. BAPNA-hydrolyzing activity of *P. gingivalis* in the presence of ions with concentrations identical to the S-PRG eluate. Four ions with concentrations identical to the S-PRG eluate were added to the reaction mixture containing BAPNA and *P. gingivalis* extract (BO_3^{3-}: 126.59mM; F^-: 6.71mM; Al^{3+}: 0.68mM; Sr^{2+}: 1.91mM). After incubation at 37℃ for 30 min, the absorbance was measured at 405 nm. *: Significant difference compared to control (0% S-PRG) ($*p<0.05$).

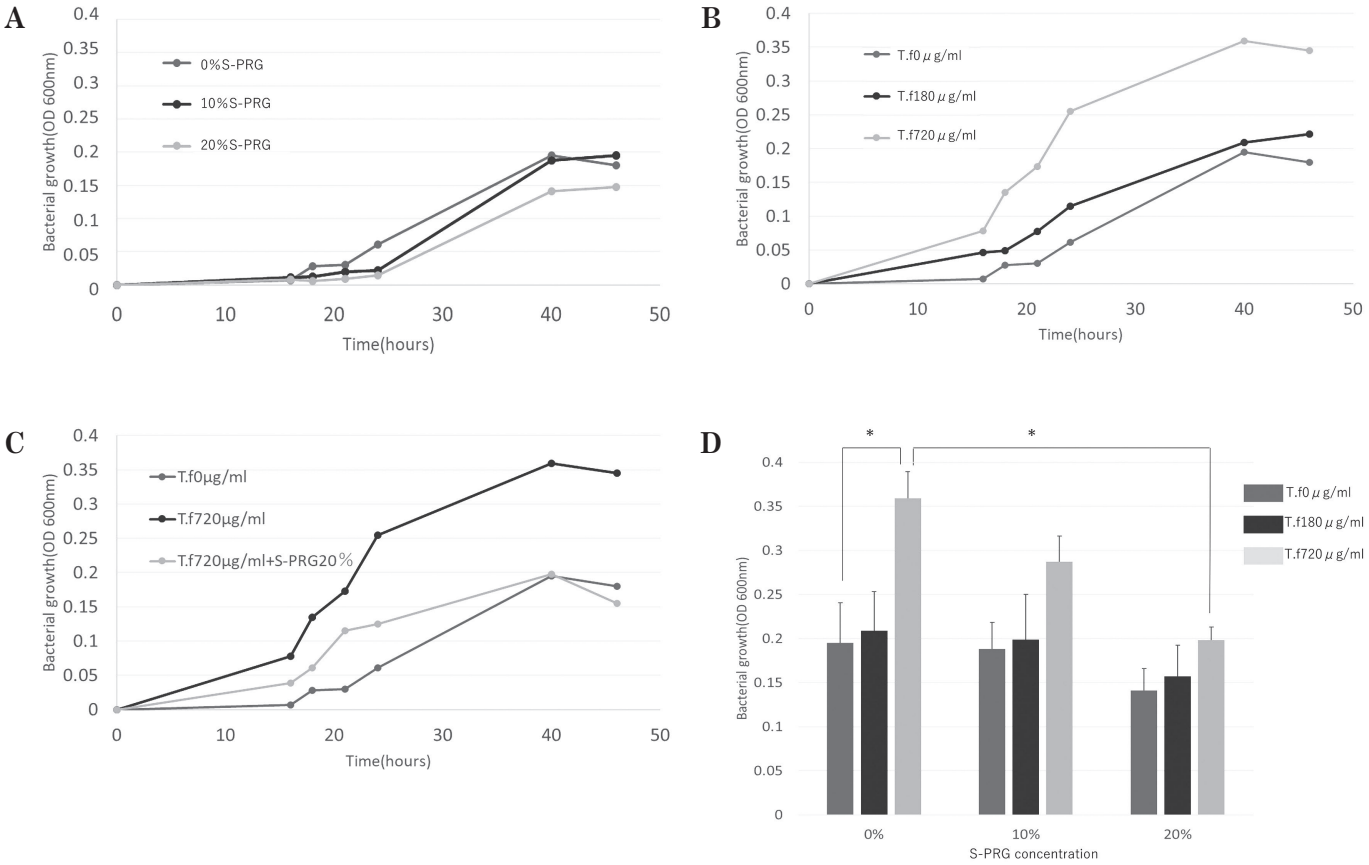

Figure 4 Effect of S-PRG eluate on the growth promotion of *P. gingivalis* by *T. forsythia* extracts.

A. Effect of S-PRG eluate on the growth of *P. gingivalis*. Zero, 10% and 20% of S-PRG eluate were added to the diluted tryptic soy broth (TSB). The growth curve of *P. gingivalis* is shown.

B. Zero, 180 μg/ml and 720 μg/ml of *T. forsythia* sonicated extracts (SE) were added to the diluted TSB. The growth curve of *P. gingivalis* is shown.

C. Zero, 720 μg/ml of *T. forsythia* SE, and 720 μg/ml of *T. forsythia* SE plus 20% of S-PRG eluate were added to the diluted TSB. The growth curve of *P. gingivalis* is shown.

D. Summary of bacterial growth at 40 min. The growth (OD 600 nm) of *P. gingivalis* after 40 h of culture under different conditions is shown. *: Significant difference between 720 μg/ml of *T. forsythia* SE (*$p<0.05$). **: Significant difference between 720 μg/ml of *T. forsythia* SE only and 720 μg/ml of *T. forsythia* SE plus 20% S-PRG eluate (*$p<0.05$).

strongest effect (Figure 2B). Suppressive effect of S-PRG eluate on protease activity of *P. gingivalis* is already known, but this is the first report which showed the importance of boron ion in inhibiting the protease activity. Boron is considered to be active in reducing proteases[33], and S-PRG may be effective as a biocompatible protease inhibitor in dental materials. Boron is also known to have an antibacterial activity in cutaneous diseases and periodontitis[34,35], and inhibits bacterial and fungal quorum sensing[36]. Since quorum sensing is a key factor in biofilm formation, boron in S-PRG eluate may exhibit both anti-biofilm and anti-protease properties.

The periodontal environment is composed of a large variety of microorganisms, and red complex bacteria are known to be the most important in the initiation and progression of periodontitis[7]. It is important to consider the effect of S-PRG eluate on bacterial interactions. *P. gingivalis* and *T. forsythia* are often recovered from the same sites of severe periodontal and endodontic lesions[37,38]. Extracts of *T. forsythia* are known to promote the growth of *P. gingivalis*[22], so we evaluated the effect of S-PRG eluate on this growth promotion. Sonicated extracts of *T.*

forsythia promoted the growth of *P. gingivalis*, but S-PRG eluate diminished this promoting effect. It is an important finding that S-PRG does not only inhibit the activity of *P. gingivalis*, but also affect the inter-bacterial interaction. Extracts of *T. forsythia* did not promote the growth of a gingipain-deficient strain of *P. gingivalis*[22], suggesting that gingipains degraded the proteins in the *T. forsythia* extract and used them as nutrients in the nutrition-reduced medium. S-PRG eluate may have inhibited the activity of the gingipains and disturbed the growth promoting effect of *T. forsythia*.

Oral malodor production is associated with many functions of periodontopathic bacteria[39], including bacterial growth, biofilm formation, and protein degradation. S-PRG eluate seems to suppress these functions, resulting in a reduction in oral malodor *in vivo*. There is another possible mechanism for oral malodor reduction, which is not associated with bacterial activities. We are now examining the possibility that S-PRG eluate make VSCs non-volatile by its buffering effect. S-PRG is widely used in the oral cavity, in composite resins, mouthguards, and removable prosthodontics. The multiple ions released from S-PRG-containing dental materials can exert their effect for a long period, which may help control chronic diseases such as periodontitis and oral malodor without affecting the oral flora.

5. Conclusion

Our findings indicate that ions released from widely-used dental materials containing S-PRG may have a beneficial effect against oral malodor and periodontitis by the following results.

1. S-PRG eluate suppressed the growth of *P. gingivalis*.
2. S-PRG eluate decreased the concentration of VSCs.
3. The most important ion in protease suppression was found to be boron ion.
4. S-PRG eluate diminished the growth promotion of *P. gingivalis* by *T. forsythia*.

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7. Conflicts of Interest

There are no conflict of interests in this work.

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原著論文

S-PRG 溶出液が歯周炎および口臭産生にかかわる細菌活性におよぼす影響

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歯周炎はいくつかのグラム陰性細菌によって引き起こされ，口臭はこれらの細菌によって産生される揮発性硫黄化合物（VSC）が関係している。本研究の目的は，歯周炎や口臭を抑制する，抗生剤や消毒剤以外の製品を特定・開発することである。多機能性表面処理ガラス（S-PRG）が，歯周炎や口臭に関連する細菌活性を阻害するイオンを放出すると仮定し研究を行った。具体的には，*Porphyromonas gingivalis* のプロテアーゼ活性，VSC 産生，および細菌相互作用に対する S-PRG の影響を調べた。その結果，S-PRG は *P. gingivalis* の増殖を抑制し，VSC の産生を減少させる傾向を示した。S-PRG 溶出液，特にホウ酸イオンは，*P. gingivalis* のプロテアーゼ活性を抑制した。*Tannerella forsythia* の菌体抽出物は，*P. gingivalis* の増殖を有意に促進したが，この効果は S-PRG 溶出液の存在下で消失した。これらの結果は，広く使用されている S-PRG 含有歯科材料から放出されるイオンが歯周炎や口臭の抑制に貢献する可能性があることを示している。