JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Platelet aggregation promoted by biofilms of oral bacteria and the effect of mouth rinses *in vitro*

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Abstract

Introduction: The purpose of this study was to observe platelet aggregation promoted by biofilms of *Streptococcus sanguinis* and *Porphyromonas gingivalis* and to evaluate the effect of two different mouth rinses on this process.

Methodology: In the first experiment, the same amount of *S. sanguinis*, *P. gingivalis*, and the *S. sanguinis* + *P. gingivalis* mixed solution was added to an equivalent amount of platelet-rich plasma (PRP). Aggregation was measured using a recording platelet aggregometer.

In the second experiment, *S. sanguinis*, *P. gingivalis*, *S sanguinis* + *P. gingivalis* mixed solutions were pretreated with either Listerine antiseptic mouth rinse or Xipayi mouth rinse for 3 minutes, 6 minutes, and 10 minutes, respectively. The same amount of solution was added to the PRP, and the inhibition of aggregation was measured.

Results: In the first experiment, *S. sanguinis* and *P. gingivalis* were able to induce platelet aggregation. The aggregation rate of *S. sanguinis* + *P. gingivalis* was significantly lower than that of either *S. sanguinis* or *P. gingivalis*.

In the second experiment, when *S. sanguinis*, *P. gingivalis*, and the *S. sanguinis* + *P. gingivalis* mixed solutions were pretreated with Listerine antiseptic mouth rinse for 3 minutes and Xipayi mouth rinse for 10 minutes, there was no significant platelet aggregation.

Conclusions: Platelets could adhere to *S. sanguinis* or *P. gingivalis*, but when *S. sanguinis* was mixed with *P. gingivalis*, the aggregation rate was reduced significantly. Treatment with Listerine antiseptic mouth rinse or Xipayi mouth rinse inhibited the ability of the bacteria to induce platelet aggregation.

Key words: *S. sanguinis*; *P. gingivalis*; Listerine antiseptic mouth rinse; Xipayi mouth rinse; platelet aggregation.

J Infect Dev Ctries 2016; 10(7):704-711. doi:10.3855/jidc.6639

(Received 24 January 2015 – Accepted 23 November 2015)

Introduction

Streptococcus sanguinis (*S. sanguinis*) is an indigenous Gram-positive bacterium that has been recognized for a long time to have a key role in the colonization of the human oral cavity. It frequently causes bacteremia and infective endocarditis (IE) [1]. Kozarov *et al*. examined 129 samples of DNA extracted from 29 atheromatous individuals for the presence of bacterial 16S rDNA sequences. *S. sanguinis* was found in approximately 20% of the samples [2]. Nakano *et al.* also found that *S. sanguinis* was detected frequently in heart valves (69%) and atheromatous plaque samples (74%) [3]. Herzgerg *et al*. discovered that *S. sanguinis* could induce platelet aggregation *in vitro* and *in vivo* [4]. *S. sanguinis*-induced platelet aggregation has been studied by a number of investigators [5-7]. *Porphyromonas gingivalis* (*P. gingivalis*) is a Gram-negative anaerobe that has long been considered to be an important pathogen associated with human chronic periodontal disease [8]. Animal experiments have shown that injections of *P. gingivalis* promote coronary artery and aortic atherosclerosis [9]. Haraszthy *et al.* examined 50 human specimens obtained during carotid endarterectomy. Hybridization of the bacterial 16S rDNA-positive specimens with species-specific oligonucleotide probes revealed that 44% of the specimens were positive for at least one of the target periodontal pathogens, and 26% were positive for *P. gingivalis* [10]. These observations indicated that *P. gingivalis* plays a role in the development and progression of atherosclerosis, leading to coronary vascular disease. *S. sanguinis* is an early colonizer of the tooth surface [11]. The G^+ streptococcal strains that initiate the colonization of the human tooth surface typically co-aggregate with each other, with saliva as the medium, to form a relatively simple ecological

membrane. These co-aggregation processes generally involve adhesin-mediated recognition of streptococcal receptor polysaccharides (RPS) [12]. Colonization of the oral cavity by *P. gingivalis* is facilitated by adherence to various oral surfaces including epithelial cells, the salivary pellicle that coats tooth surfaces, and other oral bacteria that comprise the plaque biofilm [13]. Some studies have shown that the Mfa1-SspB interaction is essential for the adherence of intact *P. gingivalis* to streptococcal cells and for the subsequent development of *P. gingivalis* biofilms on streptococcal substrates. Consistent with this, *P. gingivalis* biofilm growth exhibits the same selectivity for streptococcal species [14]. We have found that *S. sanguinis* or *P. gingivalis* alone can trigger platelet aggregation, and the combination of the two bacterial species induces less platelet aggregation and adherence to a *S. sanguinis-P. gingivalis* biofilm [15]. Currently, the main treatment of gingivitis and periodontitis is the mechanical removal of plaque and its control with drugs. Mouth rinses are used commonly in adjuvant therapy. The active ingredients of Listerine antiseptic mouth rinse are thymol, eucalyptol, methyl salicylate, and menthol. Some studies have found that oil-containing antiseptics may have additional effects on bacteria exposed to sub-lethal levels [16]. Whitaker *et al.* showed that essential-oil mouth rinse can interfere with *S. sanguinis* and *P. gingivalis* cell surface-associated activities that can induce platelet aggregation *in vitro* [17]. Fine *et al.* [18] found that rinsing with essential-oil mouth rinse has an impact on the subgingival plaque flora. Cavalca Cortelli *et al.* [19] evaluated the probing pocket depth, and plaque and gingival indices around all the teeth of 20 moderate chronic periodontitis subjects. They found that there was a greater reduction in the pocket depth, the plaque index, and the gingival index in patients using Listerine antiseptic mouth rinse, compared to the control group. The Listerine antiseptic mouth rinse group also had a significant reduction in the occurrence of *P. gingivalis* [19]. Our research has also determined that essential-oil mouth rinses have an impact on saliva and gingival plaque flora in Chinese periodontitis patients [20]. The Chinese medicine, Xipayi mouth rinse, is made by Gallic, and is effective against oral bacteria that can cause simple gingivitis and mild periodontitis. It can be used for the prevention and treatment of oral bacterial infectious diseases, and also helps to alleviate the symptoms and reduce the plaque index and the gingival bleeding index. In this study, we used the aggregometer to confirm the interaction with platelets of biofilms composed of *S. sanguinis*, *P. gingivalis*, or the combination of *S. sanguinis* and *P. gingivalis*, the rate of which was measured automatically by a computer system. We then examined the effect of pretreatment of the biofilms with Listerine Antiseptic mouth rinse and Xipayi mouth rinse on this process. The inhibition of aggregation was measured using a recording platelet aggregometer.

Methodology

Materials

Streptococcus sanguinis 133-79 was a kind gift from Professor Herzberg of the University of Minnesota. *Porphyromonas gingivalis* ATCC33277 was kindly provided by Ninth People's Hospital of Shanghai Jiao Tong University.

Bacteria culture

Streptococcus sanguinis 133-79 was grown in brain-heart infusion (BHI) medium (OXOID, Basingstoke, England) supplemented with 5% degreasing blood for 24 hours. *P. gingivalis* ATCC 33277 was grown in BHI medium (OXOID, England) supplemented with 5% degreasing blood and 0.1% vitamin K for 72 hours under anaerobic conditions, equilibrated in an atmosphere consisting of 10% CO_2, 10% H_2, and 80% N_2.

Bacterial cell treatment

Cells from 24–72-hour cultures of *S. sanguinis* or *P. gingivalis* were washed in sterile phosphate-buffered saline (PBS) and centrifuged at 1,000 rpm for 5 minutes. This process was repeated three times. The bacteria were then dispersed in sterile PBS to achieve an optical density (OD = 2.0 at 600 nm) corresponding to 2.0×10^8 cells/mL, as determined by counting in a hemocytometer [17].

Platelet treatment

Fresh human blood drawn from healthy individuals (donors had not taken aspirin or other medications in the preceding two weeks) was mixed with 3.8% buffered citrate solution (1:10), and centrifuged at 1,000 rpm for 10 minutes. The upper layer was collected and centrifuged at 1,000 rpm for 15 minutes. The upper layer was again collected and centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded, and the platelet-rich plasma (PRP) was washed with Tyrode's solution and resuspended in the same solution at a concentration of 400,000 platelets/mL.

Platelet aggregation promoted by biofilms of bacteria

S. sanguinis or *P. gingivalis* cultures with optical densities of 2 (at 600 nm) were used for the following experiments.

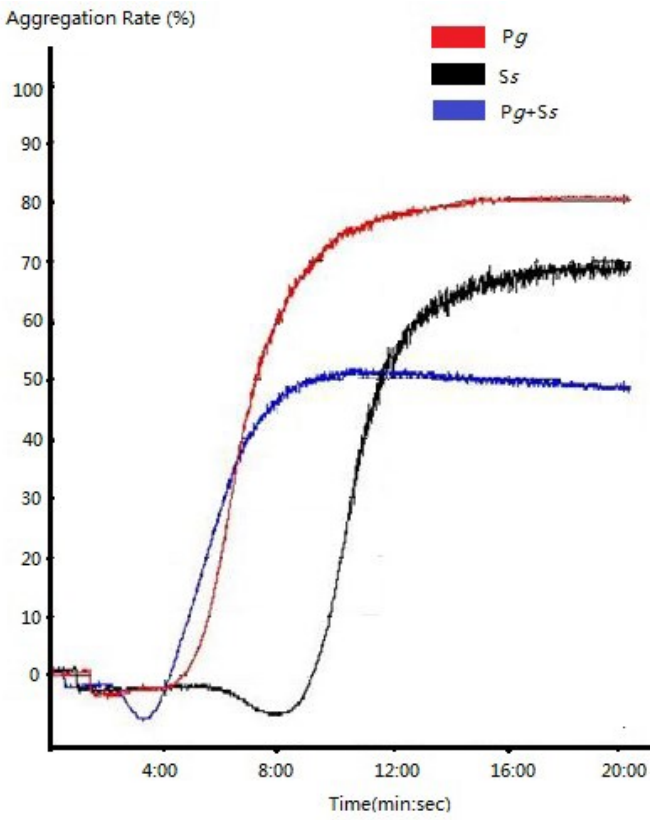
Experimental group

Fifteen microliters of *S. sanguinis* and 15 µL of *P. gingivalis* cultures were mixed for 30 minutes to ensure that the two species could interact with each other. Thirty microliters of *S. sanguinis*, *P. gingivalis*, or *S. sanguinis* + *P. gingivalis* mixed solution were added to 250 µL of PRP. Aggregation was measured using a recording platelet aggregometer.

Positive control group

Thirty microliters of adrenaline (10 µg/mL) were mixed with 250 µL PRP for 20 minutes as the positive control to confirm the ability of the platelets to aggregate [21]. Aggregation was measured using a recording platelet aggregometer.

Figure 1. Platelet aggregation promoted by biofilms of bacteria.



S. sanguinis and *P. gingivalis* were able to induce platelet aggregation. The lag times of *S. sanguinis* were significantly higher than that of *P. gingivalis* ($p < 0.05$). The aggregation rate of *S. sanguinis* + *P. gingivalis* was significantly lower than that with either bacterium alone ($p < 0.05$). The lag time of *S. sanguinis* + *P. gingivalis* was not significantly different from either *S. sanguinis* or *P. gingivalis* ($p > 0.05$)

Negative control group

Thirty microliters of sterile PBS was mixed with 250 µL PRP for 20 minutes as the negative control. Aggregation was measured using a recording platelet aggregometer.

The platelet aggregation assay was repeated three times for each bacterium.

Effect of the two antimicrobial drugs on the bacteria

Thirty microliters of *S. sanguinis*, *P. gingivalis*, and *S. sanguinis* + *P. gingivalis* mixed solution were centrifuged and resuspended in 300 µL of either Listerine antiseptic mouth rinse or Xipayi mouth rinse for 3 minutes, 6 minutes, and 10 minutes, respectively. They were then centrifuged, the supernatant was removed, and the cells were washed three times by resuspending them in sterile PBS, centrifuging, and replacing the supernatant with sterile PBS [17]. The same amount of the solutions described above (30 µL) were added to the PRP (250 µL). The inhibition of aggregation was measured using a recording platelet aggregometer.

The experiments with the bacteria were repeated three times.

Calculations, data analysis, and statistics

SPSS version 16.0 package was used for statistical analysis. The average aggregation rate was expressed as the mean ± standard error (SE) The results with the different groups were compared using one-way analysis of variance (ANOVA). Groups of independent, normal, and the variance measures data were used for the mean of multi-factor analysis of variance, using the single-factor analysis of variance (one-way ANOVA) to compare the accumulation rate between the experimental groups. $P < 0.05$ was taken as a significant difference.

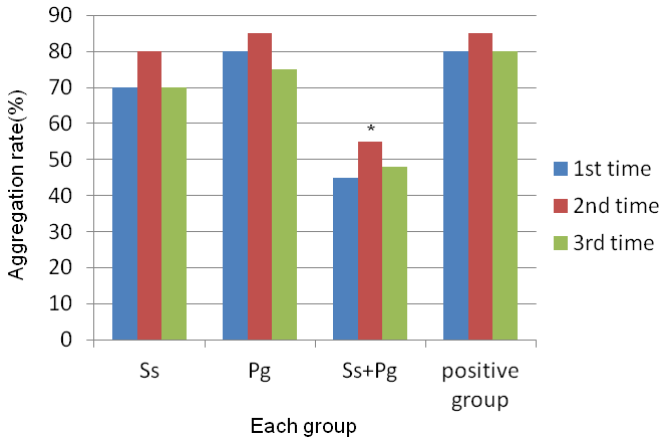
Results

Platelet aggregation promoted by biofilms of bacteria

Experimental group

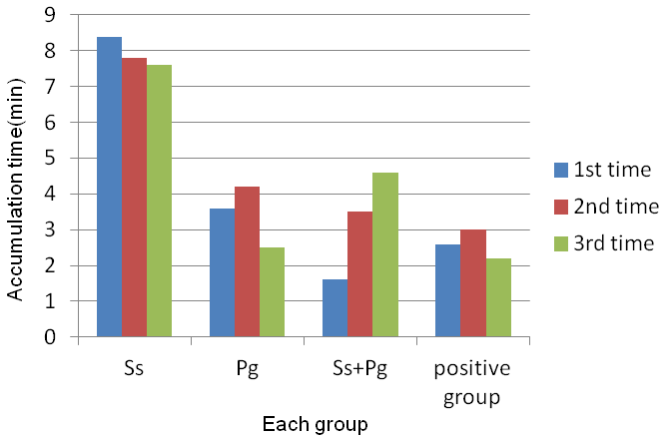
S. sanguinis and *P. gingivalis* were able to induce platelet aggregation, with mean (± SE) aggregation rates of 73.33 ± 5.77% and 80.78 ± 5.89%, respectively, and mean (± SE) lag times of 7.93 ± 0.42 minutes and 3.43 ± 0.86 minutes, respectively. The lag times of *S. sanguinis* were significantly higher than that of *P. gingivalis* ($p < 0.05$). The aggregation rate of *S. sanguinis* + *P. gingivalis* was 49.33 ± 5.13%, which was significantly lower than that with either bacterium alone ($p < 0.05$). The lag time of *S. sanguinis* + *P.*

Figure 2. Aggregation rate.



S. sanguinis and *P. gingivalis* were able to induce platelet aggregation, with mean (± S.E.) aggregation rates of 73.33 ± 5.77%, and 80.78 ± 5.89%, respectively. The aggregation rate of *S. sanguinis* + *P. gingivalis* was 49.33 ± 5.13%, which was significantly lower than that with either bacterium alone ($P < 0.05$). * indicates that compared with other groups, the aggregation rate decreased significantly, $P < 0.05$.

Figure 3. Accumulation time.



The mean (± S.E.) lag times of *S. sanguinis* and *P. gingivalis* inducing platelet aggregation was 7.93 ± 0.42 min and 3.43 ± 0.86 min, respectively. The lag times of *S. sanguinis* were significantly higher than that of *P. gingivalis* ($P < 0.05$). The lag time of *S. sanguinis* + *P. gingivalis* was not significantly different from either *S. sanguinis* or *P. gingivalis* ($P > 0.05$)

gingivalis was not significantly different from that of either *S. sanguinis* or *P. gingivalis* ($p > 0.05$) (Figure 1; Figure 2, Figure 3).

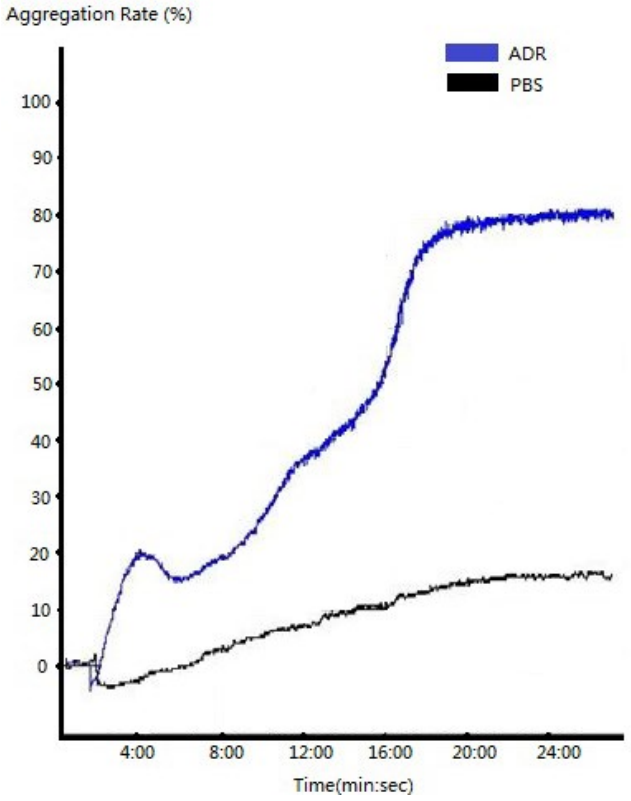
Positive control group and negative control group

The positive control group induced platelet aggregation, with a mean (± SE) aggregation rate of 81.67 ± 2.89%, and a mean (± SE) lag time of 2.64 ± 0.43 minutes. The negative control group did not induce platelet aggregation (Figure 4).

Effect of the two antimicrobial drugs on bacteria-induced platelet aggregation

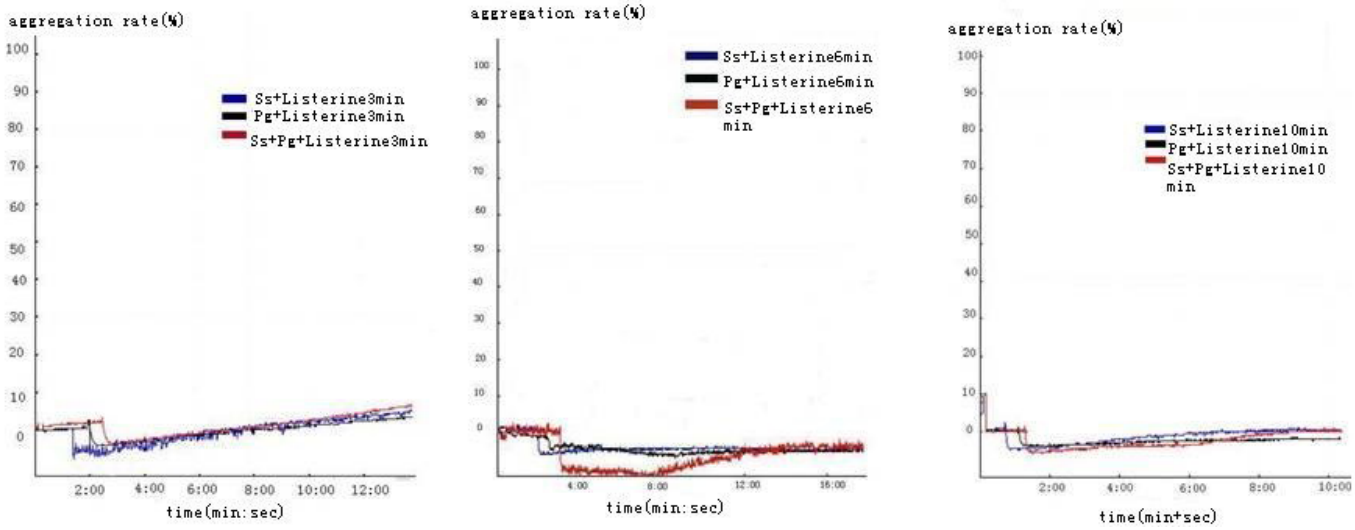
When *S. sanguinis*, *P. gingivalis*, or the *S. sanguinis* + *P. gingivalis* mixture were pretreated with Listerine antiseptic mouth rinse for 3 minutes, 6 minutes or 10 minutes, and Xipayi mouth rinse for 10 minutes, there was no significant platelet aggregation ($p < 0.01$) (Figure 5; Figure 6). After treatment with the Xipayi mouth rinse for 10 minutes, there was no effect of bacteria on platelet aggregation. When the bacteria were pretreated with Listerine antiseptic mouth rinse for 3 minutes, there was no effect on platelet aggregation.

Figure 4. Positive control group and negative control group.



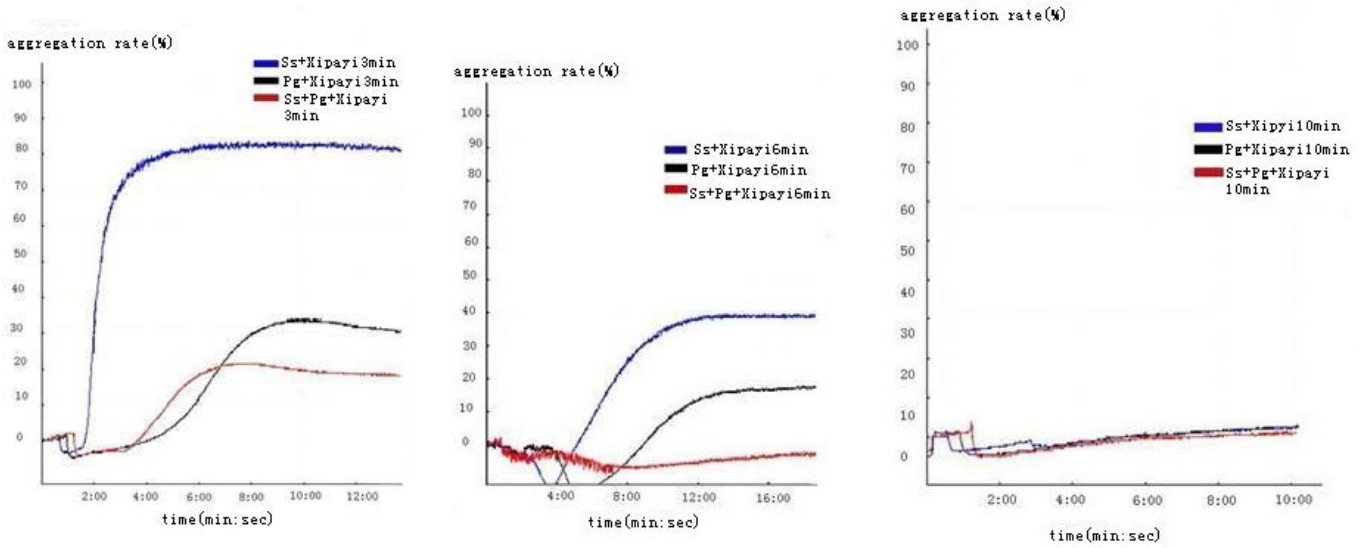
The positive control group (ADR: Adrenaline) could induce platelet aggregation. The negative control group (PBS: Phosphate buffer saline) did not induce platelet aggregation.

Figure 5. Effect of Listerine antiseptic mouth rinse on *S. sanguinis*/*P. gingivalis*/*S. sanguinis* + *P. gingivalis* for 3 minutes/6 minutes/10 minutes.



There was no significant platelet aggregation when *S. sanguinis*, *P. gingivalis*, or the *S. sanguinis* + *P. gingivalis* mixture were pretreated with Listerine antiseptic mouth rinse for 3 minutes, 6 minutes or 10 minutes ($p < 0.01$).

Figure 6. Effect of Xipayi mouth rinse on *S. sanguinis*/*P. gingivalis*/*S. sanguinis* + *P. gingivalis* for 3 minutes/6 minutes/10 minutes.



There was some effect on platelet aggregation when the bacteria were pretreated with Xipayi mouth rinse for 3 minutes and 6 minutes. There was no effect of bacteria on platelet aggregation after treatment with the Xipayi mouth rinse for 10 minutes.

Discussion

Our experiments showed that *S. sanguinis* could induce platelet adhesion and aggregation *in vitro*. *S. sanguinis* has been shown to induce platelet aggregation in a thromboxane-dependent manner, requiring adenosine diphosphate (ADP) secretion [22]. The platelet aggregation-associated protein (PAAP) expressed on the surface of inoculated cells of *S. sanguinis* promotes the accumulation of platelets onto valvular lesions [23]. Herzberg *et al.* [7] found that the components needed to promote adhesion to platelets *in vitro* and to induce subsequent platelet aggregation are present on the cell surface of certain strains of *S. sanguinis*. One of these components, PAAP, contains a determinant that is functionally and immunologically cross-reactive with the platelet-interactive domains on types I and III collagen [7]. *P. gingivalis* has received considerable attention as it is believed to be the major pathogen of adult periodontal diseases. Hemagglutination is related to the ability of *P. gingivalis* to adhere to host tissues, which is an initial step in bacterial infection. *P. gingivalis*-mediated hemagglutination has special significance, since this bacterium grows much faster in the presence of hemin than in cultures without hemin. Mutant analysis revealed that a major hemagglutinin of *P. gingivalis* is intragenically encoded by rgpA, kgp, and hagA [24]. The mechanism of *P. gingivalis*-induced platelet aggregation in PRP has been investigated. Proteinase inhibitors of Arg-gingipain (Rgp) and Lys-gingipain (Kgp) did not suppress *P. gingivalis*-induced platelet aggregation in PRP, whereas the Rgp inhibitor markedly inhibited *P. gingivalis*-induced platelet aggregation when washed platelets were used. Mutant analysis revealed that *P. gingivalis*-induced platelet aggregation in PRP depended on Rgp-, Kgp- and hemagglutinin A (HagA)-encoding genes that intragenically coded for adhesins such as Hgp44 [25]. Future studies in our laboratory will focus on the molecular mechanisms of the signal pathway in *P. gingivalis*-induced platelet aggregation, since they are poorly understood. *P. gingivalis* initially colonizes the oral cavity by interacting with organisms in supragingival plaque, such as the oralis group of oral streptococci. An important characteristic of *P. gingivalis* in colonizing the gingival crevice is its ability to adhere to other bacterial species and to crevicular epithelial cells [26]. This interaction involves the association of the streptococcal antigen I/II with the minor fimbrial antigen (Mfa1) of *P. gingivalis* [14]. *P. gingivalis* adherence to oral streptococci is multimodal and involves at least two distinct sets of adhesins and receptors [27]. *In vitro*, *P. gingivalis* adheres avidly to sessile streptococci, and once attached, it rapidly forms a biofilm comprising towering microcolonies separated by fluid-filled channels [28]. *S. sanguinis* has the ability to generate hydrogen peroxide, which is a key factor in the antagonistic interaction between *S. sanguinis* and *P. gingivalis* [29]. An important question is what will happen to the bacterial surface proteins when *S. sanguinis* adheres to *P. gingivalis*? The findings of the present study could lay the foundation for future research. Our experiments showed that Listerine mouth rinse and Xipayi mouth rinse can interfere with the ability of *S. sanguinis* and *P. gingivalis* to promote platelet aggregation *in vitro*. Listerine had a faster and stronger effect than did Xipayi. The essential-oil mouth rinse has the ability to affect cell surface-associated activities, and interfere with their main functions [17]. The mechanism for the anti-plaque activity is the disruption of the cell wall and the inhibition of bacterial enzyme systems [30]. *In vivo* and *in vitro* studies confirmed that the essential-oil components can kill oral bacteria and some opportunistic microorganisms [31], and induce extensive alterations in bacterial and fungal cell surface ultrastructural morphology following short exposures [32]. Xipayi mouth rinse liquid is an Uyghur medicine, and the composition is gallic. In recent years, studies found that gallic compounds have various pharmacological activity. They have some effects on bacterial [33], viral [34], cardiovascular, and cerebrovascular diseases [35]. Clinical observations have shown that Xipayi mouth rinse has a strong antimicrobial effect for the prevention and treatment of oral bacterial infectious diseases, that it can help relieve the symptoms, and that it can reduce the plaque index and gingival bleeding index. The two drugs investigated in this study may help to effectively prevent and control gingivitis, periodontitis, and periodontal disease, particularly in patients with atherosclerosis and/or thrombosis.

Conclusions

Platelets could adhere to *S. sanguinis* or *P. gingivalis*, but when *S. sanguinis* was mixed with *P. gingivalis*, the aggregation rate was reduced significantly. Treatment with Listerine antiseptic mouth rinse or Xipayi mouth rinse inhibited the ability of the bacteria to induce platelet aggregation, and the inhibition ability of Listerine antiseptic mouth rinse was stronger than Xipayi mouth rinse.

Acknowledgements

This work was conducted in the Laboratory of Pathology and Pathophysiology, and the Laboratory of Biomedical Engineering & Instrument Science of Zhejiang University (Hangzhou, China). This investigation was funded by the Education Department of Zhejiang Province (grant number Y201329612), the National Natural Science Foundation of China (Grant No 81371142), and 2011 China State Key Clinical Department Grants.

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Conflict of interests: No conflict of interests is declared.