In Vivo Inhibition of *Porphyromonas gingivalis* Growth and Prevention of Periodontitis With Quorum-Sensing Inhibitors

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Background: Autoinducer (AI)-2 has an important role in biofilm formation in the oral environment. Mature biofilms formed as a result of the cell-to-cell communication make it difficult to overcome periodontitis with the use of antibiotics. Previous in vitro studies suggest that quorum-sensing inhibitors (QSIs) interfere with AI-2. This study compares the QSI effects resulting from an oral inoculation of *Porphyromonas gingivalis* in an experimental animal model.

Methods: Forty-five male mice were divided into three groups (n = 15 each): 1) infection; 2) QSI; and 3) control. Infection and QSI groups received oral inoculation of *P. gingivalis*, whereas treatment with QSIs (furane compound and D-ribose) was only performed in the QSIs group. The control group was a negative control not receiving manipulation. After 42 days, mice were sacrificed, and the distance from the alveolar bone crest (ABC) to the cemento-enamel junction (CEJ) was measured by microcomputed tomography. *P. gingivalis* DNA was quantified in the soft and hard tissues around the molar teeth by real-time polymerase chain reaction.

Results: Distance from ABC to CEJ was significantly increased in the *P. gingivalis* infection group compared with the control group ($P = 0.02$) and significantly decreased in the QSI group compared with the infection group ($P = 0.02$). The QSI group contained 31.64% of the bacterial DNA count of the infection group.

Conclusion: Use of QSIs in the mice infection model showed a reduction of bone breakdown and a decrease in the number of bacteria in vivo, suggesting that QSIs can be a new approach to prevention and treatment of periodontitis. *J Periodontol 2016;87:1075-1082.*

KEY WORDS

Biofilms; models, animal; *Porphyromonas gingivalis*; quorum sensing; ribose.

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Bacteria perform particular behaviors only when living in a community and not in isolation.[1] Bacterial phenotypes depend on gene expression, which can be stimulated by cell-to-cell signaling between other microorganisms.[2] Through cell-to-cell communication, bacteria regulate the following: 1) bioluminescence; 2) secretion of virulence factors; 3) biofilm formation; 4) sporulation; 5) conjugation; and 6) pigment production, all of which can be harmful to humans.[3-5] This is conducted by the accumulation and interaction with small extracellular molecules known as autoinducers (AIs). AI-2 quorum sensing (QS) is a major QS signal molecule in which the bacteria either maintains the population density or triggers active proliferation to achieve a quorum.[1,6] In particular, AI-2 causes cell-to-cell communication between oral bacteria and plays an important role in biofilm formation.[7,8]

Infections associated with biofilm have been estimated to mediate >65% of all chronic infections in humans.[9,10] Periodontitis and dental caries are representative oral diseases that are generally acknowledged to be associated with oral biofilm.[11,12] Oral biofilm shows resistance to antibiotics. Estimates of 1,000 to 1,500 times greater resistance for biofilm-grown cells compared with planktonic grown cells have been suggested,[13]

doi: 10.1902/jop.2016.160070

and a subgingival mature biofilm needs 250 times greater concentration of antibiotics than the planktonic state for treatment.[14] Because of this resistance, it is not easy for anitbiotics to penetrate the thick structure of mature biofilms and achieve a therapeutic effect.[15]

For maturation of oral biofilm, AI-2 of *Fusobacterium nucleatum* plays a bridging role that links early colonizing commensals and late pathogenic colonizers. A previous in vitro study revealed that *F. nucleatum* AI-2 contributes to the interspecies interaction between the so-called "red complex" (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*).[16] Conversely, AI-2 of *F. nucleatum* could play an inhibitory effect on *Streptococcus oralis*.[17] The action of QS and biofilm formation has been shown to be weakened by QS inhibitors (QSIs).[18,19] Jang et al.[20] suggested in an in vitro study that two QSIs, furanone compound [(5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone] and D-ribose, were shown to significantly inhibit biofilm growth of three different species of bacteria: 1) *F. nucleatum*, 2) *P. gingivalis*, and 3) *T. forsythia*. No undesirable cytotoxic effects or induction of proinflammatory factors were observed. QSIs disrupt the signaling and colonization between bacteria and prevent increases in cell density.[20] By disrupting the biofilm, thereby making the bacteria more susceptible to traditional antibiotics, these QSIs may provide the new therapeutic approach against infections or diseases involving drug-resistant bacteria.[21] Among the QSIs, furanone compound and D-ribose were the most remarkable AI-2 inhibitors that have been discovered to date.[20,22,23] It was hypothesized that QSIs would inhibit the action of *P. gingivalis* AI-2 and reduce the biofilm formation and coaggregation and the progress of periodontitis in a mice model. To prove this hypothesis, experimental periodontitis with *P. gingivalis* inoculation was induced, and the efficacy of two QSIs, furanone compound and D-ribose, were tested on biofilm formation and the expression of periodontitis.

MATERIALS AND METHODS

Animals

All procedures were conducted following the guidelines of National Institutes of Health for the Care and Use of Laboratory Animals. The protocol for animal maintenance and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea (SNU-140618-1). Forty-five male BALB/c mice (≈8 weeks old; 200 g) were used. Animals were kept in a specific pathogen-free room in which the indoor temperature was maintained at 23 to 25°C with a 12-hour light/dark cycle.

Bacterial Strain and Growth Conditions

P. gingivalis (ATCC 33277) was grown in a brain–heart infusion medium that was supplemented with 10 μg/mL hemin[§] and 0.2 μg/mL vitamin K.[‖] Bacteria were grown in an anaerobic chamber (5% H_2, 10% CO_2, and 85% N_2) at 37°C for 18 hours.

QSIs

A furanone compound[¶] and D-ribose[#] were used as AI-2 QSIs. The furanone compound was synthesized based on the method by Manny et al.[24]

Experimental Design

A modified mouse model was used to reproduce experimental periodontitis.[25] Animals were given sulfamethoxazole (1 mg/mL)[**] and trimethoprim[††] (200 μg/mL) in their drinking water *ad libitum* for 10 days, thus inhibiting commensal bacteria. Antibiotics were administered by mixing with 463 mL deionized water. Because of light sensitivity, the water bottle was covered with aluminum foil to protect from the light. Animals were given a 4-day resting period without antibiotics after 10 days of antibiotics application.

Mice were randomized into three groups (n = 15 each): 1) infection; 2) QSIs; and 3) control. For the infection group, a 100-μL mixture of 2% carboxymethyl cellulose sodium[‡‡] (CMC) and *P. gingivalis* (5.0×10^9 cells/mL) was administered orally and applied topically to the anus at each treatment. CMC was expected to be sustained longer in the oral cavity.[26-28] For the QSI group, a 100-μL mixture of 2% CMC, *P. gingivalis* (5.0×10^9 cells/mL), and 20 μM furanone compound was orally administered and topically applied anally. D-Ribose was mixed with drinking water (50 mM D-ribose in 500 mL drinking water) for daily administration. Although D-ribose itself is water soluble, furanone needs to be mixed with alcohol, and if furanone mixed with alcohol was added to drinking water, the side effects of alcohol abuse may have surfaced.[29,30] Therefore, D-ribose was mixed with drinking water, whereas furanone with alcohol was orally inoculated by injection form with CMC. For the control group, a 100-μL mixture of 2% CMC and phosphate-buffered saline was orally administered and topically applied anally each treatment. Each group received administration of oral solution injection 10 times at 48-hour intervals by a 1-mL syringe. One additional administration was applied at 5 days before the animals were sacrificed (Fig. 1). Rodents are coprophagic in nature, and this trait can create a cycle of oral reinfection. They intake their

§ Sigma-Aldrich, St. Louis, MO.
‖ Sigma-Aldrich.
¶ Sigma-Aldrich.
# Tokyo Chemical Industry, Tokyo, Japan.
** Sigma-Aldrich.
†† Sevatrim, Swiss Pharmaceutical, Tainen City, Taiwan.
‡‡ Tokyo Chemical Industry.

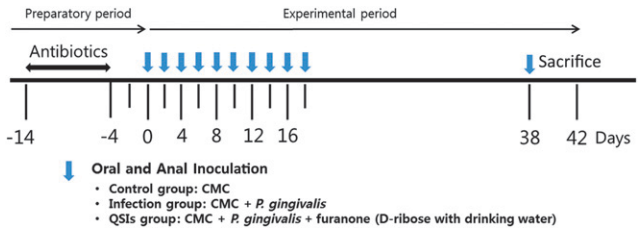

Figure 1.
Study design. Animals were given antibiotics for 10 days, with a 4-day resting period before experimentation. In the experimental period, the control group received CMC only, the infection group received CMC and P. gingivalis, and the QSI group received CMC, P. gingivalis, and QSI (furanone compound) by oral and anal inoculation. The QSI group was given D-ribose in drinking water every day. Blue arrows indicate 11 times of oral and anal inoculation.

feces, and bacteria can return to their oral cavity.[31,32] Forty-two days after the first gavage, all mice were anesthetized by intraperitoneal injection[§§][‖‖] before the animals were sacrificed. Mice were sacrificed by placing them in a CO_2 chamber to harvest maxillary and mandibular tissues.[25] Maxillary tissue and teeth were separated using a surgical saw for microcomputed tomography (micro-CT) analysis; mandibular tissues and teeth were also separated for polymerase chain reaction (PCR) analysis. For this, mandibular tissue and teeth were grinded together because biofilm could attach to the surface of the tooth.

Three-Dimensional (3D) Micro-CT Analyses of the Interproximal Area

A micro-CT scanner[¶¶] was used to examine tissues that were harvested from the maxillary molar. Tissues were fixed in place using a positioner and scanned every 0.2° for >240° in the direction of the major axis of the sample. Each specimen was scanned 1,200 times. All two-dimensional (2D) images were then scanned and saved as tif files (1,120 × 1,120 pixels). For the micro-CT data that were converted to 2D images, maxillary first molar (M1) and maxillary second molar (M2) were set in parallel,[##] and the resulting binary images were saved.

The distance from the alveolar bone crest (ABC) to the cemento-enamel junction (CEJ) of the interproximal site between M1 and M2 was measured.[***] For this measurement, the buccal surface of M1 and M2 was used as the reference point. The shortest distance from the ABC to the line connecting the adjacent CEJs between M1 and M2 was measured (Fig. 2). Among the sagittal images, the image that showed the most recession of alveolar bone was selected for measurement.

Real-Time PCR

Soft tissue, tooth, and mandibular bone were taken from the left side of each mouse. To acquire biofilm-grown cells, a tooth and soft tissue around the tooth were harvested using a surgical blade. The tissue samples were finely ground and then mixed. Next, genomic DNA (gDNA) was extracted from 0.15 g tissue using a DNA extraction kit[†††] and quantified with a spectrophotometer.[‡‡‡] Next, real-time PCR was performed on a real-time PCR system[§§§] using 100 ng gDNA. DNA levels were normalized to those of mouse GAPDH. The following mouse GAPDH primer sequences were used: 1) forward, 5′-AGG TCG GTG TGA ACG GAT TTG-3′; and 2) reverse, 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′. The following *P. gingivalis* primer sequences were used: 1) forward, 5′-TGC AAC TTG CCT TAC AGA GG-3′; and 2) reverse, 5′-ACT CGT ATC GCC CGT TAT TC-3′. Transcript was determined using a PCR kit[‖‖‖] with a program consisting of warming to 50°C for 2 minutes. Initial heat activation was at 95°C for 5 minutes. The two-step cycling was followed by: 1) 40 cycles of denaturation at 95°C for 10 seconds; 2) combined annealing at 60°C for 30 seconds; and 3) extension at 60°C for 30 seconds. A final extension was at 72°C for 10 minutes.

Real-time PCR data were analyzed with the $2^{\Delta\Delta CT}$ method.[33] This method enabled the relative levels of DNA to be compared between the QSI and infection group.

Statistical Analyses

All statistical analyses were performed,[¶¶¶] and a P value <0.05 was considered statistically significant. To estimate the mean distance from the CEJ to the ABC as assessed by micro-CT in the three experimental groups, data were analyzed with a linear-effects model with the treatment methods set as fixed factors. The linear-effects model was adjusted using a generalized least-square estimation method that accounts for heteroscedasticity.

RESULTS

Real-Time PCR

Real-time PCR was conducted for 40 cycles, and the cycles at which *P. gingivalis* and mouse GAPDH DNA were detected were recorded. Samples in which *P. gingivalis* was not detected after the completion of 40 cycles were given a value of 40. Using these parameters, mouse GAPDH was detected in 14 of 15 samples in the infection group, with *P. gingivalis*

§§ Zoletil 50, Virbac Laboratoires, Carros, France.
‖‖ 2% Rompun, Bayer Korea, Ansan, Korea.
¶¶ SkyScan-1173, Bruker-MicroCT, Kontich, Belgium.
Dataviewer program, Bruker-MicroCT.
*** CT Analyzer v.1.13.5.1, Bruker-MicroCT.
††† Bacteria Genomic DNA Extraction Kit, iNtRON Biotechnology, Seoul, Korea.
‡‡‡ ND-2000 NanoDrop spectrophotometer, Thermo Fisher Scientific, Waltham, MA.
§§§ 7500 Real-Time PCR System, Thermo Fisher Scientific, Grand Island, NY.
‖‖‖ QuantiFast SYBR Green PCR Kit, Qiagen, Hilden, Germany.
¶¶¶ R v.2.15.3, R Foundation for Statistical Computing, Vienna, Austria.

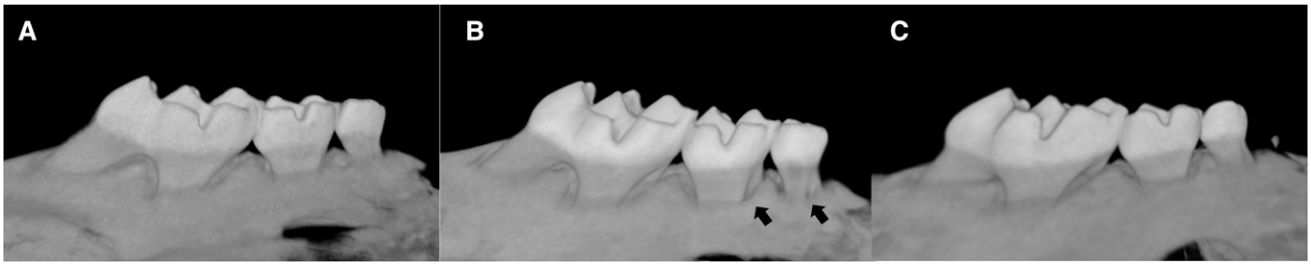

Figure 2.
3D micro-CT images at 7 weeks after infection. ***A)*** *Control group.* ***B)*** *Infection group.* ***C)*** *QSI group. In the infection group, significant bone loss was observed at the proximal and marginal areas of the maxillary molar. Otherwise, there was no significant difference between the QSI and control group.*
***** *P <0.05, statistically significant difference among control, infection, and QSI groups (hierarchical linear model with t test).*

detected in 11 samples. The mean cycle threshold (C_T) value for the mouse GAPDH was 14.97, whereas the mean C_T value for *P. gingivalis* was 35.26. Mouse GAPDH was detected in 14 of 15 samples in the QSI group, whereas *P. gingivalis* was detected in eight samples. The mean C_T value for the mouse GAPDH was 15.18, whereas the mean C_T value for *P. gingivalis* was 37.15 (Table 1). The difference in the C_T values for *P. gingivalis* and mouse GAPDH (*P. gingivalis* C_T – mouse GAPDH C_T) was calculated for each sample. The mean ΔC_T values were –20.30 and –21.96 for the infection and QSI groups, respectively. When the ΔC_T value of the infection group was corrected to 0 ($\Delta\Delta C_T$: ΔC_T of the infection group – ΔC_T of the infection group) for normalization, the $\Delta\Delta C_T$ of the QSI group (ΔC_T of the QSIs group – ΔC_T of the infection group) was 1.66. Also, when the $2^{-\Delta\Delta CT}$ method was used to compare the groups, the QSI group exhibited a reduced bacteria DNA amount of *P. gingivalis* (31.64%) compared with the infection group.[33]

Micro-CT Analyses of the Interproximal Area

Table 2 and Figure 3 summarize the distributions of bone level after the 6-week period. The mean distance from the CEJ to the ABC in the infection group was significantly longer than that of the control (CMC only) group (mean ± SE of difference: 0.0205 ± 0.0080 mm, $P = 0.02$), which confirmed that the *P. gingivalis* infection breakdown model used in the current study successfully shows breakdown of alveolar bone as expected. In addition, the mean distance from the CEJ to the ABC in the QSI group was significantly shorter than that of the infection group (mean ± SE of difference: 0.0212 ± 0.0086 mm, $P = 0.02$), suggesting that the QSIs successfully prevented alveolar bone loss (ABL) from *P. gingivalis* infection. Furthermore, the 95% confidence interval (CI) of the difference of mean distances from the CEJ to the ABC between the QSI and control group was –0.0102 to 0.0117 mm, which satisfied the predefined equivalence margin of –0.06 to 0.06 mm, demonstrating that the degree of the protection effect was as good as the negative control. 3D image reproductions are shown in Figure 4.

Table 1.

Comparison of *P. gingivalis* Level in the QSI Group Relative to the Infection Group

Group	*P. gingivalis* C_T	GAPDH C_T	*P. gingivalis* DNA Amount Relative to the Infection Group
Infection	35.26	14.97	1.0
QSI	37.15	15.18	0.3164

Aliquots of cDNA were used as templates for real-time PCR reactions containing either primers and probe for *P. gingivalis* or primers and probe for GAPDH. Each reaction contained cDNA derived from 100 ng total DNA.

DISCUSSION

To the best of the authors' knowledge, this in vivo study is the first to evaluate the effectiveness of two different AI-2 QSIs: 1) a D-ribose; and 2) a furanone compound. D-ribose and the furanone compound have been known as representative compounds of the AI-2/LuxS QS inhibition system. The AI-2/LuxS QS system is the most conserved signaling pathway for both Gram-positive and Gram-negative bacteria.[6] *P. gingivalis* also uses the AI-2 system for signaling and biofilm attachment, and expression of virulence for *P. gingivalis* can be controlled by the LuxS gene.[34,35] Recently, several QSI in vivo studies have reported results concerning subcutaneous infection,[36] *Pseudomonas aeruginosa* infection,[37] and *Staphylococcus epidermidis* infection.[38] Although combined effects of antibiotics and QSIs were evaluated, the focus was on the action of QSIs alone for regulation of periodontitis and biofilm growth inhibition.

To reproduce an inflammation around the periodontal tissue, a modified mouse infection model was used.[25] This model has been widely used to reproduce experimental periodontitis in rodents and is capable of inducing significant bone loss.[26,31,39-41] After *P. gingivalis* inoculation, the bacteria of each

Table 2.

Summary of Estimated ABC–CEJ Distances (mm) in M1-M2 Interproximal Area (± SD, n = 15)

Group	Mean	95% CI		Minimum	Maximum
		Lower	Upper		
Control	0.1232 ± 0.1486	0.1165	0.13	0.1004	0.1429
Infection	0.1438 ± 0.7872	0.1279	0.1596	0.1059	0.1911
QSI	0.122.5 ± 0.2937	0.113	0.132	0.886	0.154

group were expected to establish a mature biofilm that binds irreversibly to the surface of the pellicle layer, not to planktonic cells. To compare the groups using real-time PCR, the tooth and the surrounding soft tissue were harvested and mixed to acquire a mature biofilm. Real-time PCR and the $2^{-\Delta\Delta CT}$ methods were used for the relative comparison of biofilm load among the groups. The QSI group exhibited only 31.6% of the *P. gingivalis* DNA count compared with the infection group. It indicates that the topical use of the two QSIs reduced the amount of biofilm states' load. The natural compound (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone is known to inhibit the pathway of AI-2 and AI-1 and interacts with LuxS gene in the AI-2 QS system without affecting their growth of bacteria.[42,43] The synthetic furanone compound that is used in the current study interacts with the LuxS protein in the AI-2 QS system and has been shown to inhibit the biofilm formation of *P. gingivalis* in vitro.[20,43] The LuxS gene of *P. gingivalis* is required for synthesizing AI-2, and mutation of LuxS gene diminished AI-2 production in *P.gingivalis*.[35] D-Ribose also competed with AI-2-interacting protein RbsB or LsrB, and the biofilm growth by *Aggregatibacter actinomycetemcomitans* was reduced in the presence of D-ribose.[44] Similar to previous studies, QSI used for drinking water and topical injection in this study can interact with LuxS gene of *P. gingivalis* and induce *P. gingivalis* to be washed out on planktonic state by self-purification. Unlike antibiotics, QSIs do not affect the survival of bacteria.

From the micro-CT results, the bone breakdown of the infection group was significantly higher than the control group that received only the CMC. This suggests that inoculation of *P. gingivalis* leads to a high bacterial cell density, and the cysteine proteases Arg-gingipain and Lys-gingipain are released by *P. gingivalis*. These are two major cysteine proteinases by *P. gingivalis* associated with LuxS protein and interact with adhesion and periodontal tissue damage.[35] When the QSI group was compared with the infection group, the distance between the ABC and CEJ in the QSI group was significantly lower than the infection group. This finding suggests that D-ribose and furanone compound disrupt AI-2 biosynthesis of *P. gingivalis*. It inhibits LuxS gene expression, eventually suppressing periodontal disease. These results are consistent with some previous in vitro studies.[17,20] This may be a meaningful approach for reducing the progression of periodontitis. Because QSIs use a different mechanism to prevent bacterial growth from antibiotics, the use of QSIs can prevent the antibiotic resistance. When furanone and D-ribose were used against *F. nucleatum* AI-2, one previous study[20] that only examined single-species biofilm growth in vitro also observed a marked decrease in biofilm growth, but this

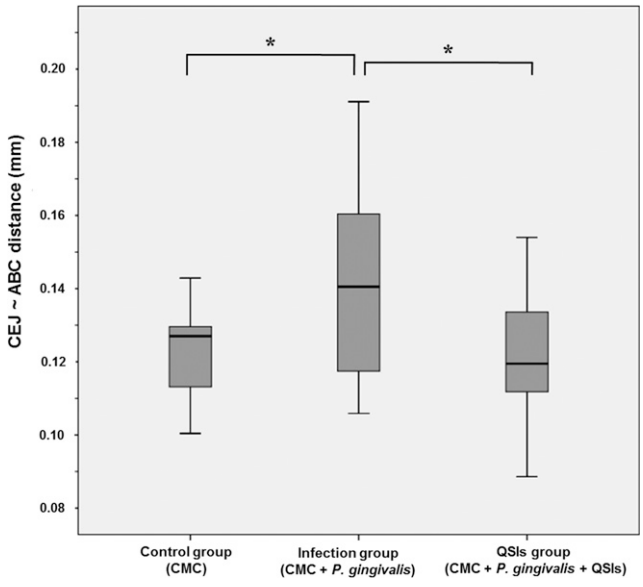

Figure 3.
Histogram showing the CEJ–ABC distance on the micro-CT results. The distance of the infection group was significantly higher than the control group (P = 0.02), and the distance of the QSI group was significantly lower than the infection group (P = 0.02). In addition, there was no significance between the control and QSI group.

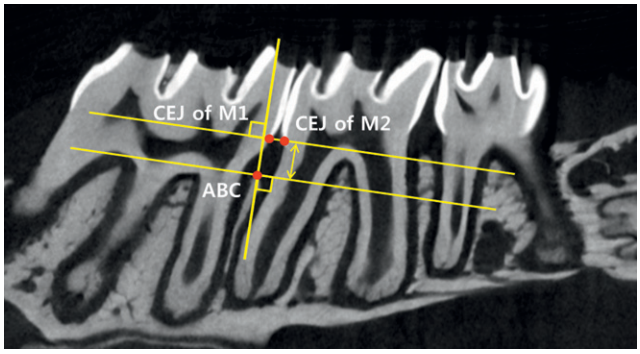

Figure 4.
Measurement method of 3D micro-CT images. A line is drawn to connect the CEJs of adjacent M1 and M2, and the shortest distance between this line and the ABC is measured.

study aims to evaluate the actions of QSIs on multispecies biofilm growth in vivo.

For statistical analysis, three sequential hierarchical statistical tests were performed as follows to control the familywise error rate to a level of 0.05. To validate a successful breakdown of the alveolar bone in the *P. gingivalis* infection model used in the current experiment, the mean ABC–CEJ distance in the infection group was compared with that of the control group. If the mean distance was significantly longer in the infection group than the control group, the mean distance in the QSI group was compared with that in the infection group to prove the beneficial effect of QSIs on the protection of ABL. If the mean distance was significantly shorter in the QSI group than the infection group, an equivalence test to compare the mean distances from the CEJ to the ABC between the QSI and control group was conducted to demonstrate that the degree of the inhibition effect was as good as the control group. According to the general statistical methods,[45] the equivalence margin was defined as half of the lower limit of the CI of the difference between the control and the infection group from a previous study that used the same breakdown model.[46] From the literature in detail, the difference of the means between the control and infection groups was 0.32 mm, and their standard errors (SEs) were 0.7 mm. Based on the independence of the two groups, the SE of the difference of the means between the two groups can be calculated as 0.10 mm, or square root of the sum of square of 0.7 mm. Thus, the 95% CI of the difference of the means between the control and infection group was estimated at 0.12 to 0.52 mm. Finally, as defined above, the equivalence margin was defined as half of the lower limit of the CI, or 0.06 mm.

In the oral cavity, a rich source of microorganisms and their dynamic interaction exist,[47] but in this study, antibiotics are administered to kill the commensal bacteria focusing only on *P. gingivalis* biofilm growth. The interactions between other bacteria, including *F. nucleatum* and *T. forthysia*, were not observed.

This is a pilot study testing a new QSI compound, and future studies will aim to optimize the concentrations and the applied frequencies or volume of QSIs and should focus on combining effects with antibiotics to the treatment of human periodontitis.

The real-time PCR was used for the relative comparison of bacterial counts between the infection and QSI groups. As a result, the DNA amount of bacteria in the QSI group was only 31.6% of the infection group. It supports that the QSIs interfere with the LuxS/AI-2 QS system and biofilm formation in vivo, and it further supports that QSIs are a new approach to the prevention and treatment of periodontitis.

CONCLUSIONS

The observations from the present study suggest that the regular application of D-ribose in the drinking water and the topical inoculation of furanone significantly interfere with biofilm growth. QSIs are expected to play an important role to interfere with the virulence expression of *P. gingivalis*.

ACKNOWLEDGMENTS

This study was supported by Grant A120757 from Korea Healthcare Technology R&D, Ministry of Health and Welfare, Seoul, Republic of Korea (to K-TK). The authors report no conflicts of interest related to this study.

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Submitted February 4, 2016; accepted for publication April 17, 2016.