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Manipulation of Autoinducer-2 Attenuates Experimental Periodontitis Induced by Mixed Infection

Autoinducer-2의 조절을 통한 복합감염원인의 실험적 치주염 완화

2018 년 02 월

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Manipulation of Autoinducer-2 Attenuates Experimental Periodontitis Induced by Mixed Infection

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2017 년 12 월

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위원
Abstract

Manipulation of Autoinducer-2 Attenuates Experimental Periodontitis Induced by Mixed Infection

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1. Objectives

Autoinducer-2 (AI-2) is a small quorum sensing (QS) molecule produced by many oral bacteria, within oral biofilm suggesting that this molecule might be responsible for signal exchange in mixed species communities. QS inhibitors (QSI) were demonstrated to prevent bone loss and the bacterial colonization of gingival tissues in a mouse model where periodontitis was induced by Porphyromonas gingivalis monoinfection.

The aim of the present study was to verify whether the inhibition of AI-2 could attenuate alveolar bone loss induced by Porphyromonas gingivalis / Fusobacterium nucleatum co-infection invivo from one side and to evaluate the effect of AI-2 inhibition
on bacterial infection and inflammatory response in the periodontal tissues from another side.

2. Methods
Periodontitis was induced in male balb/c mice (n=30) through the oral inoculation of P.gingivalis and F.nucleatum 6 times during 42 days. BMK-Q101 and D-ribose (QSIs) were AI-2 inhibitors that were administrated simultaneously with bacterial infection. Linear and volumetric modifications of interproximal alveolar bone levels were compared between groups through MicroCT. Total bacterial infection, P.gingivalis infection and pro-inflammatory cytokine gene expression in periodontal tissues were assessed with qRT PCR.

3. Results
Micro CT linear measurements showed a significant reduction of alveolar bone loss by approximately 40% in animals treated with QSIs when compared to the mono and co-infection groups (p<0.05). These findings were confirmed by volumetric measurements (p<0.05). While qRT PRC showed that total oral bacteria in the treatment group significantly decreased by 93% in gingival tissue samples when QSIs were administrated (p<0.05), no significant differences could be depicted in P.gingivalis infection and pro-inflammatory cytokine genes expression in periodontal tissues between groups.

4. Conclusion
The administration of (BMK-Q101) and D-ribose attenuated
alveolar bone loss induced by mouse co-infection. Less total bacteria colonization was observed in gingival tissues from treated animals. Further studies on the direct effect of AI-2 inhibition on biofilm in in vivo conditions are needed.

**Key Words:** Periodontitis, Host–parasite interactions, Antimicrobial(s)

*Student number:* 2015–23318
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I. INTRODUCTION

Although periodontal disease is one of the most documented polymicrobial infections, their etiology has remained elusive. At least one component of periodontitis is clear: the composition of microbial biofilm is significantly different in healthy sites compared to clinically defined disease sites. Depending on the microbial composition, the biofilm may be compatible with health or lead to disease (Marsh et al. 2003).

*Porphyromonas gingivalis* is a key microorganism in periodontitis, and its virulence in the periodontium has been demonstrated (Holt et al. 1999, Polak et al. 2012). Remarkably, in germ-free mice, *P. gingivalis* is unable to elicit disease by itself despite colonizing the host, suggesting that its pathogenicity is only expressed in the context of a community (Hajishengallis et al. 2011).

*P. gingivalis* often co-exists with other periodontopathic bacteria such as *Fusobacterium nucleatum*. A mixed infection with *P. gingivalis* and *F. nucleatum* augments the outcome of experimental periodontitis when compared to mono-infections with either bacterium (Polak et al. 2009), suggesting that the aggravation of virulence is one of the unique properties that holds mixed bacterial infection as a result of the specific synergy between infecting agents (Ebersole et al. 2014). One way to achieve this interaction is through the process in which micro-organisms sense surrounding environmental conditions and react to signaling molecules that alter their gene expression in a
density-dependent manner, so called quorum sensing (QS) (Fuqua et al. 1994).

QS is an umbrella designation that includes a collection of cell-to-cell communication signals (Withers et al. 2001). QS molecules control diverse important bacterial functions such as nutrient acquisition, redox modulation, and virulence (Shao et al. 2010). Bacteria can regulate gene expression in response to QS molecules released into the local environment by its own species or by bacteria of different species (Fong et al. 2001). Autoinducer 2 (AI-2), a small QS molecule, is considered a universal signal mediating messages among different bacterial species (Federle et al. 2003, Marsh et al. 2017). Within oral biofilm, AI-2 is produced by many oral bacteria, suggesting that this molecule might be responsible for signal exchange in mixed species communities (Frias et al. 2001, Merritt et al. 2003, Lamont et al. 1998). Jang et al. (2013a) have shown that AI-2 produced by F. nucleatum stimulates the co-aggregation and expression of adhesion molecules of P. gingivalis, Tannerella forsythia, and Treponema denticola. Herein, AI-2 mediated QS systems appear as valuable targets to prevent bacterial colonization. Natural and subsequently synthesized brominated furanones have been shown to interfere with the QS behavior of several bacterial strains (Surette et al. 1998, Ren et al. 2001). In light of the results from the in vitro experiments showing that a synthesized brominated furanone compound and D-ribose significantly reduced the biomass and depth of periodontopathogens biofilm (Jang et al. 2013a), these QS
inhibitors (QSIs) were verified in vivo. QSIs were demonstrated to prevent bone loss and the bacterial colonization of gingival tissues in a mouse model where periodontitis was induced by *P. gingivalis* monoinfection (Cho et al. 2016). However, when considering the multispecies nature of AI-2 signaling and the polybacterial infection that embodies periodontal diseases, no data has revealed the effects of these inhibitors in experimental circumstances more closely mimicking periodontitis.

The primary goal of the present study was to verify whether the inhibition of AI-2 could prevent alveolar bone loss and induced by *P. gingivalis/F. nucleatum* co-infection in vivo. The secondary goals were to evaluate the effect of AI-2 inhibition on bacterial infection of periodontal tissues of and to determine local changes of pro-inflammatory cytokines genes expression in the same animal model.
II. MATERIALS AND METHODS

QS inhibitors
In this study, two AI-2 QSIs were used: a new synthesized furanone analogue (BMK-Q101), and D-ribose. The chemical nomenclature of BMK-Q101 is 3-(dibromomethylene) hexahydroisobenzofuran-1(3H)-one and was synthesized (Park et al. 2017). Commercially available D-ribose was purchased (Tokyo Chemical Industry, Japan).

Bacteria culture
F. nucleatum (ATCC 25586) and P. gingivalis (ATCC 33277) were cultured anaerobically (10% H₂, 10% CO₂, 80% N₂) in brain heart infusion broth supplemented with hemin (10 mg/ml) and vitamin K (0.2 mg/ml) at 37°C.

Animals
Thirty 8-week-old male BALB/c mice were subjected to experiments performed in the Specific Pathogen-Free unit of the Laboratory Animal Facility at the School of Dentistry, Seoul National University, Seoul, Republic of Korea, and approved by the Institutional Animal Care and Use Committee (SNU-160609-2). Animals were kept in a room with 12h light/dark cycle and temperature varying between 23 and 25°C and housed in soft, sterile bedding and free of antibacterial
products. For feeding purposes, powdered, sterile, non-granular food was administrated to animals to prevent food impaction around gingiva, as well as sterile non-acidic water.

Mice were randomly assigned to one of the following groups:
- Group 1 (n=6): no periodontitis, no treatment.
- Group 2 (n=6): mono-infection with *P. gingivalis*, no treatment.
- Group 4 (n=6): dual infection with *P. gingivalis* and *F. nucleatum*, no treatment.
- Group 5 (n=6): dual infection with *P. gingivalis* and *F. nucleatum*, administration of QSIs.

Animal weight and general health parameters were assessed in all animals every two days throughout the experiment period.

**Induction of experimental periodontitis**

To experimentally induce periodontitis, a previously described mouse model was adapted for the present study (Baker et al. 2000, Polak et al. 2009). All animals first received sulfamethoxazole (1 mg/mL) (Sigma–Aldrich, USA) and trimethoprim (200 mg/mL) (Sevatrim; Swiss Pharmaceutical, Taiwan) in their drinking water *ad libitum* for 10 days in order to reduce native flora and support colonization of the pathogenic bacteria. Following a 4-day resting period without antibiotics, mice were infected according to the following protocol (Fig. 1):

- In groups of mono-microbial infection by either *P. gingivalis* or *F. nucleatum* (groups 2 and 3), an inoculum of \(10^{10}\) CFU in 100 µl of 2% PBS in 2% carboxymethyl cellulose (CMC) was directly administrated inside the oral cavity.
In the dual-infection group (group 4) and treatment group (group 5), the mice were orally administrated a consortium of *P. gingivalis* (5x10^9 CFU/ml) and *F. nucleatum* (5x10^9 CFU/ml) prepared in 100µl of 2% PBS and thoroughly mixed with 2% CMC. In the control group, the mice received only 2% PBS in 2% CMC. Oral gavage was performed 30 minutes after viable *P. gingivalis* and *F. nucleatum* were directly harvested from culture suspension and added to the innoculum. Using a 1 mL syringe, each animal received six inoculations at a 2 day-intervals during 2 weeks. Following each inoculation, mice were held without food or water for 30 minutes to minimize elimination of the inoculant from the oral cavity (Cantley et al. 2009).
Fig. 1. Schematic diagram depicting timing of *in vivo* experimental design.

After administration of antibiotics for 10 days (grey line), specific germ-free mice were orally inoculated 6 times every 2 days during 12 days (red dots). Furanone compound (20 μM) diluted in 0.0% ethanol (EtOH) and mixed to the oral inoculum 2 μM /mL. D-Ribose was added to the drinking water (50 mM /500 mL). Animals were sacrifice by after 42 days.
**QSIs treatment**

To investigate their effects on experimental periodontitis, QSIs were administrated to mice from group 5. Furanone compound BMK-Q101 (20 μM) diluted in ethanol (EtOH 0.02%) and added to the oral inoculum containing a mixture *P. gingivalis* and *F. nucleatum* to 2 μM/mL. D-ribose was mixed with the drinking water (50 mM D-ribose in 500 mL of drinking water) and replaced every 2 days.

**Tissue collection and preparation**

At the completion of the study (day 42), mice were sacrificed with an overdose of an intraperitoneal injection of tiletamine – zolazepam (Zoletil; Virbac, France) and xylazine (Rompun; Bayer Korea, Korea). Heads were separated he mandibular and maxillary portions of the skulls were dissociated. Oral cavity soft tissue was collected from each animal by sharp dissection of mandibular buccal gingiva and oral mucosa. Individual soft tissue samples were snap frozen in liquid nitrogen and stored at −80°C until tissue assays were performed. Harvested maxilla samples were fixed in 4% paraformaldehyde for 24 hours and stored in 70% ethanol at room temperature until they were scanned.

**Quantification of alveolar bone loss**

Maxillae were scanned using a Skyscan 1173 High Resolution Micro-CT Scanner (Skyscan, Bruker, Belgium). Tissues were fixed in place using a positioner and scanned every 0.3° for >240° in the direction of the major axis of the sample. The x-ray...
generator was operated at 90 kVp of beam current at 88 µA and an image resolution of 15.98 µm. Each specimen was scanned 1,200 times. All two-dimensional (2D) images were then saved as tiff files (1,120 x 1,120 pixels). The images were reconstructed with a specific software (NRecon v1.6.9.8; SkyScan, Bruker-MicroCT, Kontich, Belgium) in all the spatial dimensions, and then oriented and saved in sagittal slices (Data Viewer v1.4.4; SkyScan, Bruker-MicroCT, Kontich, Belgium). CTAnalyzer software (Version 1.15.4.0+, SkyScan) was then used to open the images. Measurements were performed twice at 3-week-intervals by a masked trained examiner for linear and volumetric evaluation of alveolar bone loss in right and left inter-proximal areas between maxillary first and second molars (M1-M2) and between maxillary second and third molars (M2-M3) (Park et al. 2007).

For linear measurements, three sagittal sections were chosen for each interproximal area and the shortest distances from the alveolar bone crest (ABC) to the line connecting cement-enamel junction (CEJ) between M1-M2 and M2-M3 were measured (Park et al. 2007).

For volumetric measurements, three-dimensional regions of interest (ROI) were selected according to morphological landmarks. First, two-dimensional contours were drawn regularly at 15 continuous scan slices (i.e. 0.3 mm apical to the roof of the furcation). The measured area included the buccal alveolar bone around the mesio-buccal and disto-buccal roots of the second molar without the furcation (Polak et al. 2009). The trabecular morphometry was analyzed within the ROI to determine the
percentage of trabecular bone volume/total volume (BV/TV%) of the buccal bone plate.
Linear and volumetric measurements were carried out on maxillary molars from right and left sides in all mice (n=6 in each group).
**Fig. 2.** Methods for linear and volumetric measurements on micro CT reconstructions

A—A line is drawn to connect the cement–enamel junctions of adjacent M1 and M2 (left and right red dots respectively), and the shortest distance between this line and the ABC (green dot) is measured. B—ROI for bone volumetric analysis (dashed area) included vestibular alveolar bone from the mesial root of the maxillary first molar to the distal root of the third molar.
Analysis of gene expression by quantitative Real Time PCR

In the gingival specimens from the dual-infection \((n=6)\) and treatment \((n=6)\) groups, bacterial gDNA and total RNA were extracted using a genomic DNA extraction kit (G-Spin, iNtRON, Korea) and total RNA extraction kit (RNA-Spin, G-Spin, iNtRON, Korea), respectively, and quantified on a spectrophotometer (NanoDrop, Thermo Fisher Scientific, USA). cDNA synthesis was carried out from the extracted RNA using a cDNA reverse transcription kit (Power cDNA, G-Spin, iNtRON, Korea). \textit{P. gingivalis}-specific 16S rRNA gene primers, \textit{F. nucleatum}-specific 16S rRNA gene primers, and universal 16S rRNA gene primers were used to quantify \textit{P. gingivalis}, \textit{F. nucleatum}, and the total number of bacterial genes recovered from the gingival tissues, respectively. Additionally, the gene expression of interleukine-\(1\beta\) (IL-\(1\beta\)), interleukine-6 (IL-6), and tumor necrosis factor \(\alpha\) (TNF \(\alpha\)) were evaluated in the gingival samples (Table 1). Quantitative PCR was conducted using a QuantiFast SYBR Green PCR Kit (Qiagen, USA) in the duplicated gingival specimens. Bacteria gDNA and pro-inflammatory cytokine gene expression were normalized to universal 16S rRNA genes and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes in each sample, respectively.
### Table 1. Primers sequences and melting temperatures

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’−3’)</th>
<th>Reverse (5’−3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal</td>
<td>5’−GAATTTCATGACGCTAGAC−3’</td>
<td>5’−TTAAGCAGATCTGCTAG−3’</td>
<td>67°</td>
</tr>
<tr>
<td><em>F. gingivalis</em></td>
<td>5’−CTTGGACTGATGCGCGAG−3’</td>
<td>5’−AGGGAAGAGGTGGTTTTCACCA−3’</td>
<td>60°</td>
</tr>
<tr>
<td><em>F. nucleatum</em></td>
<td>5’−CAACCATTACCTTAACCT−3’</td>
<td>5’−ATTGAGCTTACTGAGGGAGAT−3’</td>
<td>60°</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’−AGGGCATGGTTG−3’</td>
<td>5’−TTAGACCAAGATTTTGAG−3’</td>
<td>59°</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’−AAGGAAGAAGCA−3’</td>
<td>5’−TGGGGAACCT−3’</td>
<td>61°</td>
</tr>
<tr>
<td>TNFα</td>
<td>5’−AAAGCATCTGAGCGCCATGTTG−3’</td>
<td>5’−CATAGATAGTTGGCTCATA−3’</td>
<td>57°</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’−GACAACTTTGGCATTG−3’</td>
<td>5’−ATGCAAGGATGTTCTG−3’</td>
<td>54°</td>
</tr>
</tbody>
</table>
Statistics

Data were analyzed using GraphPad Prism software 6 (GraphPad; San Diego, CA, USA).

For *a priori* sample size calculation, by assuming a bone loss difference of 0.1mm and a standard deviation of 0.03mm (Maekawa *et al.* 2017), a total of 30 animals (6 mice per group) was decided to achieve a statistical power of 80%.

For the comparison of data on bone loss, one-way repeated measure analysis of variance (ANOVA) was applied to test significance of differences between groups. If results were significant, intergroup differences were tested for significance using Student’s *t* test and the Bonferroni correction for multiple testing.

In addition, $2^{-\Delta\Delta CT}$ method was used to compare relative levels of genes in tissue samples analyzed with qRT PCR and differential gene expression was tested using the Student *t* test. Statistical significance was set at $p<0.05$. 
III. RESULTS

Animals
Across the duration of the experiment, no significant loss of weight was noted in mice. In addition, no other adverse events were observed. All animals survived upon the termination of the experimental protocol.

Inhibitory effects on alveolar bone loss cooperatively induced by *P. gingivalis* and *F. nucleatum*
A representative profile of alveolar bone loss secondary to intraoral infection is shown in Figs. 3 and 4. Linear measurements showed that dual inoculation by *P. gingivalis* and *F. nucleatum* resulted in bone loss that was significantly more severe in comparison to vehicle-inoculated mice (*p*<0.01) (Fig. 3-II-A). When AI-2 inhibitory compounds were added to the mixed infection, a significant reduction of alveolar bone loss by approximately 40% was observed in comparison to the positive control group (*p*<0.05) (Fig. 3-II-A). The net bone loss, calculated by subtracting the total alveolar bone loss of the negative control group from that of the infected groups, was also significantly reduced in mice where AI-2 inhibitory compounds were added to the infection inoculum and drinking water (Fig. 3-II-B). Similarly, volumetric measurements (Fig. 4-II) showed that co-infection
caused a significant decrease in bone tissue in the fraction of the ROI compared to the non-infected mice \((p<0.01)\), but was prevented by the administration of QS inhibitors \((p<0.05)\). These findings were confirmed by the residual bone volume measurements (Fig. 4-II). Bacterial co-infection caused an average decrease of bone tissue in the fraction of the ROI compared to the non-infected mice \((p<0.01)\), but was prevented by the administration of QS inhibitors \((p<0.05)\). Similarly, the micro-architecture parameters of trabecular bone in the selected ROI showed an enhancement in the bone quality of mice treated with QS inhibitors. A significant increase of trabecular number in the treatment group compared to the positive control group from one side \((p<0.05)\), and a decrease of trabecular separation from another side \((p<0.05)\) purport the prevention of alveolar bone loss in QS inhibition group.
Fig. 3. **Alveolar bone loss – Linear measurements:** Coinfection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* induced bone loss that was prevented when QSIs were administrated.

I: Representative sagittal sections from right hemi-maxilla sampled in control group (A), *Porphyromonas gingivalis/Fusobacterium nucleatum* dual infection group (B) and quorum sensing inhibitors group (C) analyzed by micro computed-tomography. Yellow and red arrows indicate the cement-enamel junction and bone levels respectively on mesial and distal aspects of the septum between the maxillary first and second molars.

II: Micro-CT analysis A– Differential alveolar bone loss between control group (Sham), dual infection group (Pg+Fn) and QSIs group (Pg+Fn+QSI). AI–2 inhibition compounds significantly
reduced bone loss in comparison to mono and mixed infection groups. Bone loss distances are represented as CEJ–ABC length. **B** - Net bone loss, showing the total ABC–CEJ distance of each mono/mixed infection and QSIs group minus that of the control group. As indicated, administration of AI–2 inhibition compounds (Pg+Fn+QSI) resulted in less bone loss compared to control group (Sham).

Linear bone loss was calculated in three selected sagittal sections. Measurements were carried in left and right interproximal area between first and second molars; and second and third molars in each maxilla from of all mice (n=6 per group). Duplicate evaluation of micro CT linear measurements performed by one blinded examiner at a 2 week-interval allowed an intra-examiner reliability of 0.974. The results are expressed as the mean ± standard error. Data were analyzed by ANOVA followed by Student’s t test for inter-group comparison. Statistically significant differences are indicated as p<0.05 and p<0.01.

Pg: P. gingivalis; Fn: F. nucleatum; QSI: BMK–Q101, D–ribose.
Fig. 4. Alveolar bone loss – Volumetric measurements: Administration of QSI maintained an interproximal residual bone volume similar to control group although dual-infection.
I: Buccal (A, B, C) and occlusal-apical (D, E, F) views of right hemimaxilla of sham-infected (A, D), Porphyromonas gingivalis/Fusobacterium dually infected mice with (B, E) or without (C, F) quorum sensing inhibitors as reconstructed by the micro-computed tomography. In occlusal-apical views (D, E, F), automatic segmentation of the teeth from the alveolar bone was performed.

II: Bone microarchitecture analysis: A– Residual bone volume and B– its distribution between calculated sites. C– Trabecular thickness, D– Trabecular number and E– Trabecular separation. Volumetric bone loss was calculated in left and right interproximal area between first and second molars; and second and third molars in each maxilla from of all mice (n=6 per group). Duplicate evaluation of micro CT volumetric measurements performed by one blinded examiner at a 2 week-interval allowed an intra-examiner reliability of 0.963. The results are expressed as the mean ± standard error. Data were analyzed by ANOVA followed by Student’s t test for inter-group comparison. Statistically significant differences are indicated as p<0.05 and p<0.01; Lower case letters indicate statistical difference with sham group (a: p<0.05; b: p<0.01)).

Pg: P. gingivalis; Fn: F. nucleatum; QSI: BMK-Q101, D-ribose; M1, M2, M3: Maxillary first, second and third molars.
Bacterial infection of periodontal tissues

To confirm mice infection by inoculated periodontopathogens, samples from gingiva and alveolar mucosa were analyzed using qRT–PCR. Table 2 summarizes the changes triggered by QSIs on bacterial genes amounts in dual infection and treatment groups. Mean values and standard deviations of cycle numbers at which P. gingivalis and total bacteria 16s rRNA gene amplification reached the threshold value of fluorescence intensity (CT) are presented. Total oral bacteria in the treatment group significantly decreased by 93% when QSIs were administrated ($p<0.05$) (Fig. 5). Opposite of F. nucleatum, P. gingivalis was constantly detected in the gingival samples in the co-infection and treatment groups. A slight reduction of the P. gingivalis specific gene was observed in the treatment group compared to the co-infection group without reaching statistical significance ($p>0.05$).
Table 2. QS inhibitors trigger changes in the bacterial load of gingival tissues analyzed by qPCR.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Infection group $\pm$ S.D.</th>
<th>Treatment group $\pm$ S.D.</th>
<th>Fold induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria (Universal)</td>
<td>20.10 ± 0.28</td>
<td>23.66 ± 2.42</td>
<td>0.07*</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>34.61 ± 0.78</td>
<td>34.56 ± 0.91</td>
<td>0.91</td>
</tr>
<tr>
<td><em>F. nucleatum</em></td>
<td>Undetected</td>
<td>Undetected</td>
<td>N/A</td>
</tr>
<tr>
<td>GAPDH</td>
<td>15.477 ± 0.38</td>
<td>15.31 ± 0.48</td>
<td>1</td>
</tr>
</tbody>
</table>
Fig. 5. Bacterial levels in periodontal tissues – QSIs trigger changes in bacterial infection of tissues analyzed by qRT PCR.

A– P. gingivalis DNA levels were similar in tissues samples from infected mice treated or not with QSIs. B– When QSIs were orally administrated, significantly less total bacteria DNA were detected. Genomic DNA levels of P. gingivalis and total bacteria were calculated in periodontal tissues (gingiva and alveolar mucosa) sampled surrounding right and left mandibular molars in each mouse from mixed infection (n=6) and QSIs (n=6) groups at day 42 using qRT PCR. Mean DNA amounts calculated relative to co-infection genes levels are represented with SE. Statistically significant differences are indicated as p<0.05.

Pg: P. gingivalis; Fn: F. nucleatum; QSI: BMK-Q101, D-ribose.
Proinflammatory cytokine gene expression in gingival tissues

Since inflammation is mainly mediated by cytokines, the gene expression of Il-1β, Il-6 and TNFα was investigated by PCR in gingival samples from mice challenged by *P. gingivalis* and *F. nucleatum* with or without QS inhibition (Fig 6). When QSIs were administered, IL-6 and TNFα displayed a decrease of 1.06 and 1.13-fold changes respectively compared to the non-treated mice. IL-1β increased by 1.14-fold in the treatment group compared to the dual-infection controls which was not statistically significant (*p>*0.05).
**Fig. 6. Cytokines genes expression in gingival tissues from infection and treatment groups.**

Less genes expression was observed for TNFα and IL-6 cytokines in treatment group when compared to mixed infection group albeit no statistical difference.

*Pg: P. gingivalis; Fn: F. nucleatum; QSI: BMK-Q101, D-ribose.*
IV. DISCUSSION

The overall objective of this study was to preclinically explore the potential of AI-2 manipulation to prevent multispecies biofilm-induced periodontitis. For this purpose, in a mouse model of periodontitis induced by mixed infection, the attenuation of alveolar bone loss and proinflammatory cytokine genes expression by AI-2 inhibitory compounds was hypothesized, as well as the modulation of bacterial infection of periodontal tissues.

Furanone and D-ribose were tested in the present study. Emerging evidence supports the capacity of furanones and D-ribose to prevent the growth of biofilm, eventually by periodontopathogens, although their mechanism of action has not been elucidated. Lönn-Stensrud et al. (2007) based on in vitro experiments conducted on oral streptococci, suggested that the interference of furanones with inter-bacterial communication resulted from a competition with AI-2 due to structural similarities (Ren et al. 2001, Blehert et al. 2003). Recently, Jang et al. (2013a) also showed a similar mechanism to explain AI-2 inhibition by a synthetic furanone compound. The authors reported a critical inhibition of biofilm formation when the three periodontopathogens of *P. gingivalis*, *F. nucleatum*, and *Treponema*...
denticola were cultured in the presence of either synthetic furanone or another QSI compound, D-ribose. The competitive binding of D-ribose to AI-2 receptors has been also proposed to explain the inhibition of bacterial biofilm (Armbruster et al. 2011, Jang et al. 2013a, Wang et al. 2016). Shao et al. (2007) showed, in Aggregatibacter actinomycetemcomitans, that ribose competes with AI-2 for the same binding site of their receptors, RbsB and LsrB, thus interfering with AI-2-dependent phenotypes such as biofilm growth. Although the known AI-2 receptors, LuxP, LsrB and RbsB are, yet, lacking in almost all oral bacteria, given the widespread production and response to AI-2 in oral biofilms, additional AI-2 receptors in oral bacteria are likely to exist (Kolenbrander et al. 2010) where AI-2 molecules as well as their analogues could bind.

Here, BMK-Q101, a newly synthesized furanone, was chosen due to its improved properties to prevent biofilm growth of oral bacteria in comparison to a reference furanone compound previously investigated (Jang et al 2013a, Cho et al. 2016). This assumption was validated through in vitro experiments demonstrating that BMK-Q101 not only significantly inhibits the AI-2 activity of F. nucleatum ATCC 25586 – a major player of biofilm maturation (Jang et al. 2013b, Ryu et al. 2016) – but also impairs biofilm growth of P. gingivalis ATCC 33227 and F. nucleatum ATCC 25586 (unpublished data). Because a concentration of 2µM induces neither bactericidal effect nor
cytotoxicity against host (Park et al. 2017), it was decided for the present study. Additionally, D-ribose was combined to the furanone to potentialize AI-2 inhibition in the animal model used in this experiment. Since furanone is soluble in alcohol and not water, furanone was not mixed with the drinking water (Cho et al. 2016) and was orally administrated through CMC as a vehicle to avoid any side effects of alcohol abuse in mice.

To experimentally inoculate periodontal pathogens into the oral cavity of animals, protocols including diet, ligature and oral infection by oral gavage have been proposed. Infection of the oral cavity by topical administration of *P. gingivalis* is a well-established model (Graves et al. 2012) and has been previously used to explore the beneficial modulation of periodontitis by furanone and D-ribose (Cho et al. 2016). Recognizing the polybacterial nature of periodontal disease and that AI-2 is, in essence, exchanged between various species among biofilm (Kolenbrander et al. 2010), a mouse model in which periodontitis is induced by mixed infection was created to verify if BMK-Q101 and D-ribose could modulate disease manifestations. Taking advantage of the present study findings, demonstrating that, in vitro, the exchange of AI-2 between *P. gingivalis* ATCC 33227 and *F. nucleatum* ATCC 25586 governs their biofilm growth, the same strains were used to orally co-infect mice. A significant periodontal breakdown was observed in mixed-infection
group. However, when the furanone compound BMK-Q101 and D-ribose were administered to the mice orally co-infected by *P. gingivalis* and *F. nucleatum*, micro CT linear and volumetric measurements displayed significantly less alveolar bone loss, corroborating the results from the previous *in vivo* experiment where furanone and D-ribose, orally administrated in mice mono-infected with *P. gingivalis*, reduced bone loss (Cho *et al.* 2016) Simultaneous inoculation of *P. gingivalis* and *F. nucleatum* was reported to amplify periodontal tissue breakdown and inflammation in mice when compared to mono-infection by either bacterium alone (Polak *et al.* 2012, Polak *et al.* 2009, Polak *et al.* 2013). In such infection, the increased virulence of *P. gingivalis* and *F. nucleatum* stems from the synergistic inter-connection between these two bacteria allowing the protection against acid attacks or satisfying the need for carbohydrates (Takahashi *et al.* 2003, Diaz *et al.* 2002). These interactions deeply rely on the proximity between the two bacteria (Polak *et al.* 2012), which is mostly sustained by co-aggregation (Bradshaw *et al.* 1998). In this regard, AI-2 signal exchange has been shown to be a crucial parameter for proteins implicated in the co-aggregation between *P. gingivalis* ATCC 33227 and *F. nucleatum* ATCC 25586 such as RgpA (Jang *et al.* 2013a). Thus, the attenuation of periodontal breakdown in QSIs group could be hypothesized to arise from the interference of BMK-Q101 and D-ribose with the AI-2 signaling, resulting in disruption of bacterial synergism and virulence.
impairment. In a mouse model of mixed-infection, as previously demonstrated in mono-infection model (Jang et al. 2013a), manipulation of AI-2 signal through oral administration of QSI s seems to impair alveolar bone loss.

If the composition shift of subgingival bacteria, orchestrated by AI-2, is a critical determinant of the disease state of periodontal tissues, periodontal pathogens are, however, poor inducers of inflammatory mediators (Lamont et al. 1995, Ji et al. 2010). A burgeoning pool of evidence indicates that bacterial invasion into gingival tissues is a key event in the initiation of periodontal tissues inflammation (Ji et al. 2015, van der Velden et al. 2017). The presence of bacteria within the tissue would recruit leukocytes to the site of infection leading to alveolar bone loss. Previous in vitro and vivo trials revealed that, in \textit{P. gingivalis}, AI-2 regulates the expression of stress response genes such as \textit{htrA}, which contributes to bacterial invasion of epithelial cells and bacterial survival in mouse models of infection (McNab et al. 2003, Yuan et al. 2008, Saito et al. 2008). Consistent with this, Cho et al. (2016) reported that in an experimental periodontitis mouse model induced by \textit{P. gingivalis} mono-infection, AI-2 inhibitors led to a decrease approximating 70% of the \textit{P. gingivalis} DNA amounts in tissues. Nonetheless, in the present study, \textit{P. gingivalis} infection did not exhibit any difference between QSI s-treated and untreated mice. A plausible reason for the
discrepancy with the \textit{in vivo} trial by Cho \textit{et al.} (2016) is the influence of the synergistic partnership resulting from \textit{P. gingivalis} \textit{– F. nucleatum} mixed–infection. \textit{P. gingivalis} invasive abilities of host cells are significantly greater when host cells are co–infected with \textit{P. gingivalis} and \textit{F. nucleatum} (Saito \textit{et al.} 2008, Saito \textit{et al.} 2009) hiding therefore an eventual inhibitory action exerted by QSIs on \textit{P. gingivalis} invasion. Interestingly, at the opposite of \textit{P. gingivalis}, total bacteria DNA, in the present study, displayed a substantial decrease of 93\% in the tissues sampled from mice treated with QSIs. In a clinical study (Abusleme \textit{et al.} 2013), the comparison of periodontal microbiota between healthy and periodontitis patients revealed a negative correlation of \textit{P. gingivalis} with total bacterial load suggesting that the properties of the overall biofilm community contributed more directly to periodontal pathogenesis than the abundance of the pathogen. Because \textit{P. gingivalis} exploits various mechanisms to subvert the host innate immune response (Polak \textit{et al.} 2013, Ji \textit{et al.} 2015), \textit{P. gingivalis} itself may not induce enough inflammation to cause alveolar bone loss. At low colonization–levels, \textit{P. gingivalis}–induced alveolar bone loss is dependent on the invasion of periodontal tissues by commensal bacteria (Hajishengallis \textit{et al} 2011, Ji \textit{et al.} 2015). Incorporating the findings from the present study, QSIs could reestablish bacterial communities within biofilm that are favorable for periodontal health, allowing the reduction of total bacteria invasion of
periodontal tissues. With a similar abundance of *P. gingivalis* in treated and untreated mice, pathogenic potential of commensal bacteria may be eventually reduced by the administration of QSIs. Nevertheless, considering the available data, no conclusion could be drawn on the mechanism by which alveolar bone-loss could be attenuated with QSIs. Further investigations of oral biofilm are necessary to provide clarifications on the mechanism by which QSIs are able to modulate periodontitis manifestations.

In an attempt to explain the underlying mechanism behind the attenuation of disease severity, further focus was laid upon the host response to mixed infection in the present study. Since inflammation is mainly mediated by cytokines, the gene expression of IL-1β, IL-6 and TNFα was investigated. The assumption that these proinflammatory cytokines could be influenced by QSIs originates from the findings reported by Scheres and colleagues (2015). While *P. gingivalis*, either as components or a whole cell, is known to induce a range of pro-inflammatory cytokines *in vitro* and *in vivo* (Gemmel *et al.* 1998, Kinane *et al.* 2001), a genetic mutation of QS components (*luxS* gene) in *P. gingivalis* drastically reduces IL-6 and IL-1β responses by the host (Scheres *et al.* 2015). This suggests that QS signaling may be also involved in the direct interaction between *P. gingivalis* and periodontal tissues in an AI-2 dependent fashion (Sheres *et al.* 2015). The inhibition of AI-2 signal could be therefore expected
to reduce the expression of pro-inflammatory cytokines in the present study, whereas no statistical difference was seen between the treatment and infection groups. These results could stem from the complexity of bacterial interaction following mixed infection for the animal model used in this study. Polak et al. (2013) showed that the mixture of live P. gingivalis and F. nucleatum in a mice subcutaneous chamber model induced a substantial attenuation of cytokine levels compared to mono-infection by either bacterium. Another plausible explanation is the resolution phase of periodontitis at which the gingival tissues were sampled (Ebersole et al. 2014). Pro-inflammatory cytokines are more prone to be released at the acute phase of the disease which may explain the comparable cytokine gene expression found in the gingival specimens. Therefore, no conclusion could be drawn on the effect of QSIs on proinflammatory cytokine expression. It will be a matter for future studies to examine cytokines release at an earlier stage of periodontitis in order to unveil any modulation exerted by QSIs.
V. CONCLUSION

Similar to the mono-infection model, periodontitis induced by oral infection with multiple periodontopathogens that exchange AI-2 could be modulated by the manipulation of QS signaling. The main outcome of this in vivo study was the attenuation of alveolar bone loss induced by mouse co-infection when a new furanone compound (BMK-Q101) and D-ribose were administrated. We concluded that QSIs had a significant protective effect on alveolar bone loss which may be linked to a modification of bacterial colonization in periodontal tissues from treated mice. Robust evidence on the effects of AI-2 manipulation is still lacking. The direct effect of AI-2 inhibition on biofilm in in vivo conditions needs to be investigated and correlated with modifications to the host response. In addition, whether the effects seen in this study were the result of QSIs’ anti-inflammatory abilities is yet to be determined.
VI. REFERENCES


Autoinducer-2의 조절을 통한 복합감염원인의 실험적 치주염 완화

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1. 연구목적

Quorum sensing(QS)라는 세포 간 통신 신호의 수집을 포함하는 명칭이다. QS 분자들은 세균의 다양한 중요한 기능, 예를 들면 영양소 획득, Redox 조절, 독성 등을 조절한다. 구강 바이오필름 내에선 Autoinducer-2 (AI-2)라는 작은 QS 분자가 다양한 구강 세균에 의해서 만들어지는데, 이는 여러 종 간의 신호교환을 책임진다고 여겨진다. QS 억제제들은 Porphyromonas gingivalis 단독감염으로 치주염을 발생시킨 쥐 모델의 치주조직에서 박테리아의 집단형성과 뼈흡수를 방지시켰다. 그러나, AI-2 신호기전이 다양한 종에서 공통적이라는 점과 치주염을 유발시키는 다중미생물 감염에 의한 치주질환들을 고려했을때, 그 어떠한 자료도 바이오필름감염에 의한 치주염을 재현한 환경에서 다중감염 원 억제제들의 효과를 밝히지 못했다.

이 연구의 우선적인 목표는 AI-2의 억제를 통해서 in-vivo 환경에서 Porphyromonas gingivalis / Fusobacterium nucleatum 공동감염에 의한 치조골흡수를 완화할 수 있는지를의 여부검사였다. 두번째로는, 같은
동물모델의 치주조직에서 AI-2 억제에 의한 세균감염과 염증반응을 평가하는 것이었다.

2. 연구방법
수컷 balb/c 쥐에서 42 일동안 6 번 P.gingivalis 와 F.nucleatum 를 구강 접종하여 치주염을 유도하였다. AI-2 억제제들인 BMK-101 과 D-ribose (QSIs)는 박테리아 감염과 동시에 주어졌다. Micro CT 를 사용해서 각 그룹 간 치간치조골의 직선적 및 용량적 변화를 측정하고 비교하였다. 치주조직에서 총 박테리아량, P. gingivalis, 그리고 전염증성 시토카인 유전자 발현량 등을 qRT PCR 을 통해서 측정했다.

3. 연구결과
직선적 측정결과를 통해 P. gingivalis 와 F. nucleatum 의 이중접종이 vehicle-접종된 쥐들보다 훨씬 더 심각한 뼈흡수를 보인 것을 확인하였 다(p<0.01). 복합감염된 조직에 AI-2 억제제가 첨가되었을 때, 양성조절 그룹에 비해서 치조골 흡수가 약 40%씩이나 감소된 것을 확인하였다 (p<0.05). 용량적 측정을 통해서는 감염되지 않은 쥐에 비해서 복합적감염이 뼈흡수를 심각하게 감소시켰지만(p<0.01), QSI 투여를 통해서 예방된 것을 확인하였다(p<0.05). 뼈의 마이크로적 구성의 변화를 보았을 때, treatment 그룹에서 trabecular separation 이 treatment 그룹에서 positive control 그룹에서보다 유의미하게 높았고 (p<0.05), 다른 면에서 trabecular number 가 treatment 그룹에서 positive control 그룹에서 보다 유의미하게 높았고 (p<0.05), 다른 면에서 trabecular separation 이 treatment 그룹에서 positive control 에 비해 낮아진 것을 확인하였는데(p<0.05) 이는 QSI 그룹이 치조골흡수 예방한다는 것을 지지했다. qRT PCR 결과에 따르면, QSI 가 조직에 추가되었을 때 treatment 그룹에서 모든 박테리아의 총량이 93%나 준 반면(p<0.05), 치주조직에서 P. gingivalis 의 양이나 전염증성 시토카인 유전자 발현량은 그룹 간 큰 차이를 보이지 않았다.
4. 결 론
단독감염 모델에서와 유사하게, AI-2 를 교환하는 다수의 치주질환균이 발생시킨 치주염도 AI-2 신호교환을 조절하므로써 완화시킬 수 있었다. 이 실험의 가장 큰 수확은 새로운 furanose compounds 인 BMK-Q101 와 Dribose 가 첨가되었을 때 치조골소실이 준 것을 확인한 것이다. AI-2 조절이 가지는 효과에 대한 강력한 증거는 아직 부족하다. AI-2 억제가 in-vivo 환경에서 바이오필름에 미치는 직접적인 영향은 연구되어야 하고 숙주반응의 변화와 연관되어져야 할 것이다.

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