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Xylitol Mitigate Neutrophil Inflammatory Response Against *Porphyromonas gingivalis* Infection

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Periodontitis is generally a chronic disorder characterized by breakdown of tooth-supporting tissues, producing dentition loss. *Porphyromonas gingivalis (P. gingivalis),* a Gram-negative anaerobic rod, is one of the major pathogens associated with periodontitis. Neutrophils are first line defense cells in the oral cavity that play a significant role in inflammatory response. Xylitol is a known anti-caries agent and has anti-inflammatory effects. In this study, we conducted experiments to evaluate anti-inflammatory effects of xylitol on *P. gingivalis* infected neutrophils for possible usage in prevention and treatment of periodontal infections. *P. gingivalis* was intraperitoneally injected and peritoneal lavage was collected for cytokine determination. For *in vitro* study, neutrophils were collected from mouse peritoneal cells after zymosan injection or bone marrow cells. Neutrophils were stimulated with live *P. gingivalis* and ELISA was used to determine the effect of xylitol on *P. gingivalis* induced cytokine production. IL-1β, IL-6, TNF-α concentration and neutrophil population in the peritoneal lavage was increased in *P. gingivalis*-infected mouse. Peritoneal cells infected with live *P. gingivalis* revealed significantly increased production of IL-1β, IL-6 and TNF-α at multiplicity of infection of 10. Neutrophils from bone marrow and peritoneal lavage revealed increased production of IL-1β, IL-6 and TNF-α. Xylitol significantly mitigated *P. gingivalis* induced cytokine production in neutrophils. Findings indicate that xylitol is an anti-inflammatory agent in neutrophils infected with live *P. gingivalis*, that suggests its use in periodontitis management.

Key words: *P. gingivalis*, neutrophil, xylitol, inflammation, periodontitis

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Introduction

Inflammation is crucial for host defense against invading pathogens [1]. Periodontitis is a chronic inflammatory disease induced by infection of major periodontopathogens, such as *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola, and Tannerella forsythia* [2,3]. In response to infection, inflammatory immune cells such as monocytes and neutrophils are recruited in periodontitis tissues and produce cytokines and chemokines which in turn activate adaptive immune response [4].

Neutrophils are the main leukocytes recruited to the gingival crevice [5], where neutrophils create a barrier against the growing bacteria biofilm. Neutrophils at the gingival crevice is thought to prevent bacteria form invading the underlying tissues [6]. However, in pathologic conditions such as chronic periodontitis, neutrophils have been suggested to be associated

with the excessive immune responses [7]. The numbers of neutrophils in inflamed periodontal tissues correlate with the severity of the lesions [8], and hyperactive oral neutrophils are found in refractory periodontitis [9].

Cytokines are secreted by immune cells and are involved in modulating inflammatory responses. Although cytokines play protective roles in eliminating the infected bacteria, overproduction of inflammatory cytokines are thought to be relevant to the periodontal destruction [10,11]. Thus, modulating excessive cytokine production may be beneficial in developing effective therapeutic approach.

Xylitol is a low-calorie sugar substitute, which is considered to be non-fermentable by oral bacteria. Previously, we have reported that xylitol has anti-inflammatory effect by inhibiting cytokine production induced by *P. gingivalis* lipopolysaccharide in Raw 264.7 cells [12]. However, the anti-inflammatory effects of xylitol on *P. gingivalis* infection against neutrophils remains to be elucidated.

The purpose of our study was to investigate anti-inflammatry effects of xylitol against *P. gingivalis* infection in neutrophils for future application of xylitol in chronic periodontitis.

Materials and Methods

Bacterial culture

P. gingivalis (strain 381) were grown in gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical, Tokyo, Japan), which contained hemin (5mg/mL) and 3-phytyl-menadione (vitamin K, 0.5mg/ml) at 37°C in an anaerobic chamber in an atmosphere containing 90% N_2, 5% H_2, and 5% CO_2. An optical density (OD) of 1.0 (650 nm) was determined to correlate to $1x10^9$ colony forming units/mL. To prepare the bacteria for infection, an overnight culture was diluted to an OD 650 nm of 1.0 in GAM broth. The bacteria were washed and resuspended in RPMI media, and was used to infect the neutrophils at a multiplicity of infection (MOI) of 10, 20 and 50.

Animal study

The study was conducted on 6-week-old male mice of C57BL/6J (Koatech, Pyeongtaek, South Korea). The animals were kept in standard conditions: temperature 20 ± 2°C, with *ad libitum* access to the consumption of water and food. Bone marrow cells were collected from femoral bones and treated with RBC lysis buffer (QIAGEN, Dusseldorf, Germany) for 5 min to remove erythrocytes. To recruit neutrophils to the peritoneal cavity, sterile peritonitis was induced by intraperitoneal (i.p.) zymosan (0.1 mg/mouse, Sigma, Saint Louise, MO) injection. Peritoneal cells were collected either 3 hrs or 18 hrs after zymosan injection. To retrieve peritoneal cells, peritoneal cavity was washed with 5 ml of ice cold PBS containing 10% FBS.

Flow cytometry analysis

For flow cytometric analysis of cell populations in the peritoneal cavity, cell suspensions were washed and stained with anti-mouse mAbs CD11b-PE/Cy7, Ly6G-PerCP/Cy5.5 and F4/80-APC/Cy7 (all from Biolegend) for 30 min at ice. Data were acquired on FACSVerse (BD Biosciences, San Jose, CA) and analyzed with Flowjo software (TreeStar, Ashland, OR, USA).

Neutrophil isolation and Cell treatment

Neutrophils were isolated using mouse neutrophil isolation kit (Biolegend, San Diego, CA) following manufacturer's instruction. The neutrophil cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and were cultured at 37°C in 5% CO_2 incubator. Neutrophils were pretreated with 1%, 2% or 5% xylitol (Danisco Sweeteners Oy, Kotka, Finland) for 30 min and were infected with *P. gingivalis* for 24 h.

Cytokine analysis

The amounts of TNF-α, IL-6 and IL-1β released into the culture media after *P. gingivalis* infection were analyzed by using an ELISA kit purchased from Biolegend (San Diego, CA, USA). Cytokine level were measured by the manufacturer's instruction. The plates were read in an ELISA reader (Tecan, Männedorf , Switzerland) at 450/570 nm.

Statistics

Statistically significant differences between samples were analyzed with an unpaired, one-tailed Student's t test. The data are shown as the mean ± SD. A p value of < 0.05 was considered statically significant.

Results

P. gingivalis induces recruitment of neutrophils and induces inflammatory cytokine production.

To examine if *P. gingivalis* recruit neutrophils and induce inflammatory response, *P. gingivalis* was i.p. injected at various

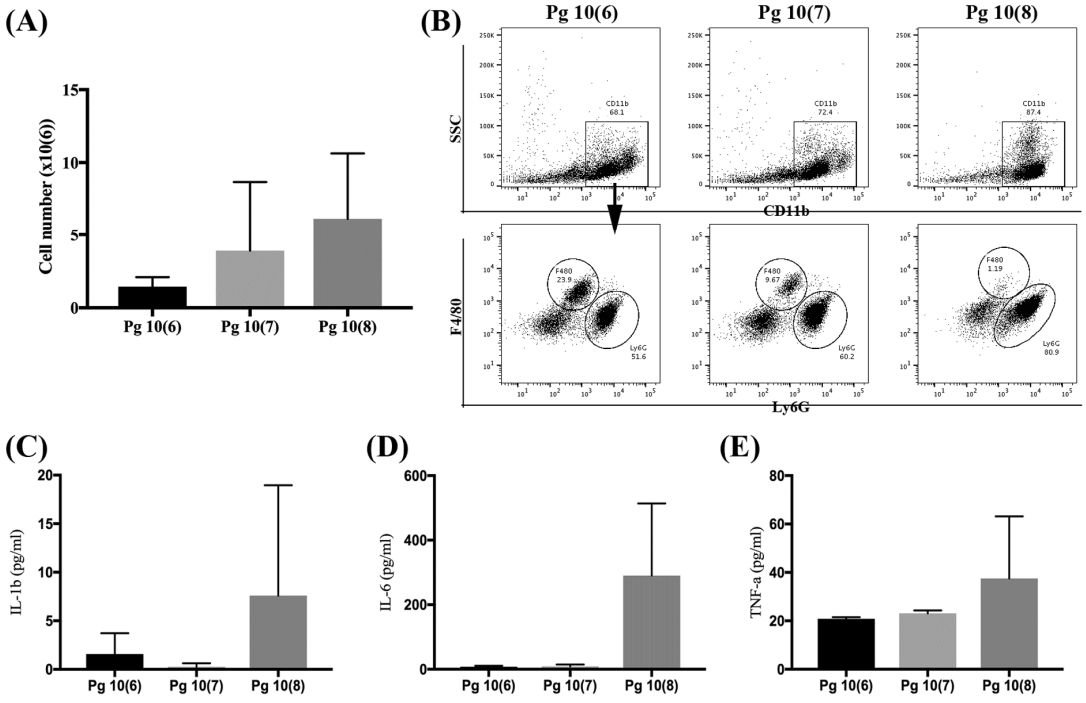

Fig 1. *P. gingivalis* i.p. injection recruit neutrophils and induce inflammatory cytokine production. (A) Total peritoneal cell number. Live *P. gingivalis* were treated at various numbers and peritoneal cells were collected after 3 hrs. (B) Characterization of peritoneal cells. Collected peritoneal cells were stained with CD11b, Ly6G and F4/80 to characterize the cells. (C) IL-1β (C), IL-6 (D) and TNF-α (E) in the peritoneal fluid were determined by ELISA. Each group consists of at least 3 mice.

concentrations. *P. gingivalis* infection showed increase of neutrophil population in the peritoneal cavity as low as $1x10^6$ infection (Fig. 1A and 1B). Also, *P. gingivalis* infection increased IL-1β, IL-6 and TNF-α production in peritoneal cavity (Fig. 1C, 1D, and 1E). Thus, *P. gingivalis* infection recruits neutrophil to the peritoneal cavity and induce inflammatory cytokine production.

P. gingivalis–induces cytokine production in neutrophils isolated from bone marrow and peritoneal cavity.

To test if *P. gingivalis* infection induce inflammatory cytokine response, bone marrow cells were challenged with *P. gingivalis* for 24 hrs. As shown in Fig. 2, the infection of bone marrow cells by *P. gingivalis* induced production of IL-1β, IL-6 and TNF-α as low as MOI 10. Thus, MOI 10 was used for further studies.

Since bone marrow cells and peritoneal cells contain mixed population of cells, neutrophils isolation kit was used to isolate purified neutrophils. When *P. gingivalis* was challenged to the isolated neutrophils, there were some characteristic cytokine production depending on the origin of the neutrophil. Bone marrow derived neutrophils showed increased production of

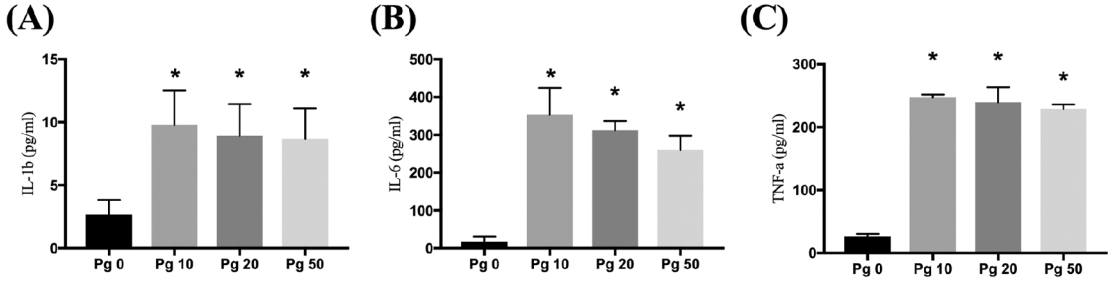

Fig 2. Live *P. gingivalis* infection induce inflammatory cytokine production in bone marrow cells. Mouse bone marrow cells were infection with *P. gingivalis* (MOI 10, 20, and 50 for 24 hr) and IL-1β (A), IL-6 (B) and TNF-α (C) were determined by ELISA. * vs uninfected cells, $p<0.05$.

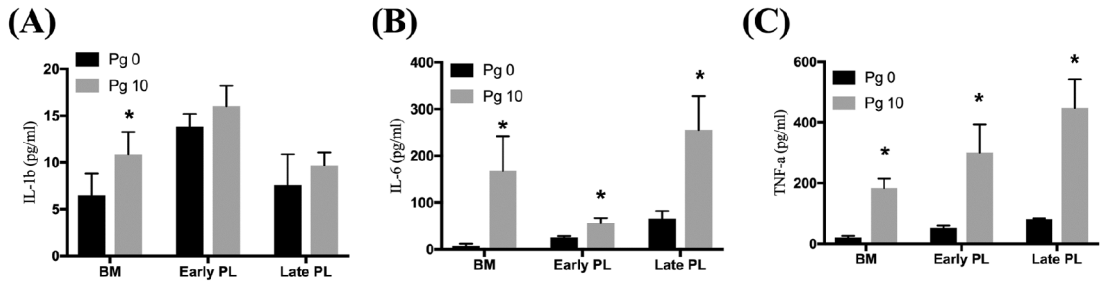

Fig 3. Live *P. gingivalis* infection induce inflammatory cytokine production in isolated neutrophils. Neutrophils were isolated from bone marrow (BM) or peritoneal cavity following zymosan i.p. injection at 3 hr (early PL (peritoneal lavage)) or 18 hrs (late PL). Isolated neutrophils were infected with *P. gingivalis* at MOI 10 for 24 hrs and IL-1β (A), IL-6 (B) and TNF-α (C) were determined by ELISA. * vs uninfected cells, $p<0.05$.

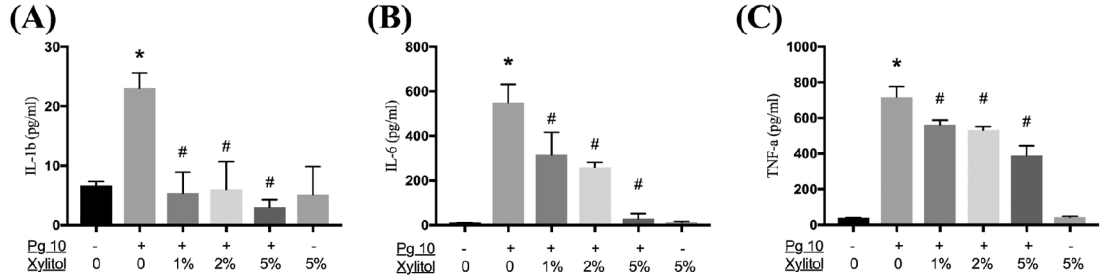

Fig 4. Effect of xylitol against *P. gingivalis* infection in neutrophils. Xylitol was pretreated for 30 mins and mouse bone marrow cells were infected with *P. gingivalis* (MOI 10 for 24 hr). IL-1β (A), IL-6 (B) and TNF-α (C) were determined by ELISA. * $p<0.05$. * vs uninfected cells. # vs *P. gingivalis* infected cells.

IL-1β, IL-6 and TNF-α ,while peritoneal derived neutrophils from both early stage (3 hr post zymosan injection) and late stage (18 hrs post zymosan injection) showed only increased IL-6 and TNF-α production (Fig. 3). In early stage peritoneal neutrophils, the baseline IL-1β was elevated compared to other neutrophils while it showed decreased production of IL-6 (Fig. 3A and Fig. 3B). Late stage peritoneal neutrophils showed highest TNF-α production (Fig. 3C). Taken together, *P. gingivalis* infection induced inflammatory cytokine production in various stage of neutrophils.

Xylitol mitigates *P. gingivalis*–induced cytokine production in neutrophils isolated from bone marrow.

Finally, we tested if xylitol is effective against *P. gingivalis* infection in neutrophils. Xylitol pretreatment significantly suppressed the production of the IL-1β, IL-6 and TNF-α at the tested concentrations (Fig. 4). Thus, xylitol showed anti-inflammatory effect on live *P. gingivalis* infection in neutrophils.

Discussion

Periodontitis, which is one of the major causes of adult tooth loss, is closely related to chronic inflammation caused by periodontopathogens including *P. gingivalis* [13,14]. Although inflammatory cytokine responses induced by bacterial infection play protective roles in eliminating the pathogen, overproduction of inflammatory cytokine may induce tissue destruction, such as attachment loss, collagen destruction, and bone resorption [3,14,15]. Thus, tight regulation of cytokine production is required for the management of periodontitis.

Neutrophils are the main leukocytes recruited to the gingival crevice [5], and they are thought to prevent bacteria from invading the underlying tissues [6]. To better understand the neutrophils response to *P. gingivalis* infection, live *P. gingivalis* was i.p. injected to the mouse and neutrophil recruitment and cytokine production was determined. *P. gingivalis* infection induced increase of neutrophil population in the peritoneal cavity and induced IL-1β, IL-6 and TNF-α production in the peritoneal cavity.

Since there are mixed population of cells in the peritoneal cavity, further experiments were preformed to examine if the inflammatory cytokines were produced by neutrophils. Because bone marrows are well known to be rich in neutrophils [16], bone marrow cells were challenged with *P. gingivalis* at various

MOI and cytokine productions were determined. Finally, isolated neutrophils infected with *P. gingivalis* showed increaed in IL-1 β, IL-6 and TNF-α production.

Excessive inflammatory response has been proposed to be detrimental by maintaining inflammation that may lead to tissue destruction [8,9]. Activated neutrophils can produce numerous inflammatory cytokines including IL-1β, IL-6, IL-12, and TNF-α [17,18]. TNF is a cytokine that influences many elements such as cellular infiltration, cytokine production, and bone resorption. In addition, TNF can induce neutrophil respiratory burst and up-regulate the expression of adhesion molecules, cytokines and chemokines [19-21]. IL-6 is a multifunctional pro-inflammatory cytokine that is synthesized in response to infection, and has a wide range of biological activities including antibody production, T cell activation, B cell differentiation, and osteoclast activation [22]. IL-1β can activate the release of other proinflammatory cytokines such as TNF and IL-6, and induce a Th17 cell differentiation [15,23]. Our result also support that *P. gingivalis* can induce neutrophils to produce IL-1β, IL-6 and TNF-α.

It has been reported that neutrophils are not homogeneous and that circulating neutrophils can show significant differences in parameters, such as phagocytosis, protein synthesis and oxidative metabolism [24]. When neutrophil transmigrate out of the blood vessels, its interaction with different components of venular wall structures which induces a number of functional and phenotypic changes in neutrophils including increased respiratory burst or neutrophil extracellular trap formation [25]. To examine if there is any features in inflammatory response depending on the origin of the neutrophils, neutrophils were prepared from bone marrow or peritoneal cavity after zymosan injection. Some features were observed suggesting that neutrophils were not homogeneous. Although nerutrophils derived from bone marrow and peritoneal cavity both showed increased production in IL-6 and TNF-α, IL-1β production was only significantly increased in bone marrow derived neutrophils. Taken together, transmigration and activation should have induced functional changes to the neutrophils.

Xylitol is well known to inhibit bacterial growth and metabolism [26]. In addition, xylitol can inhibit the production of inflammatory cytokines induced by *P. gingivalis* LPS and live *P. gingivalis* in macrophages [12,27]. Thus, we hypothesized that xylitol could mitigate the production of inflammatory cytokines induced by live *P.gingivalis* in neutrophils. In this study, we showed that xylitol effectively inhibits the production of IL-1β, IL-6 and TNF-α, which were induced by live *P. gingivalis* infection in mouse neutrophils. These results suggest that xylitol has an anti-inflammatory effects against live *P. gingivalis* infection, and in turn, the possible use of xylitol as an anti-inflammatory agent to control chronic periodontitis can be suggested. Additional studies are required to determine whether xylitol exerts the anti-inflammatory effects not only *in vitro*. It should be important to examine anti-inflammatory effects of xylitol in animal models to support the role of xylitol in periodontitis *in vivo*.

In summary, mouse infected with *P. gingivalis* induced neutrophil recruitment and inflammatory cytokine production. Isolated neutrophils infected with live *P. gingivalis* produced IL-1β, IL-6 and TNF-α. The pretreatment with xylitol significantly decreased the production of cytokines. These findings suggest that xylitol acts as an anti-inflammatory agent against live *P. gingivalis* infection, highlighting its potential clinical use in the prevention or treatment of periodontitis.

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Conflict of interest

The authors have no financial conflicts of interest.

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