JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

EFFECT OF ROSELLE CALYX EXTRACT ON GINGIPAIN ACTIVITY, PRODUCTION OF INFLAMMATORY CYTOKINES, AND ORAL BACTERIAL MORPHOLOGY

*Herastuti Sulistyani, Mari Fujita, Futoshi Nakazawa**

Address(es): Professor, Futoshi Nakazawa,
Health Sciences University of Hokkaido, School of Dentistry, Department of Oral Microbiology, 1757 Kanazawa, Ishikari-Tobetsu, Hokkaido 061-0293, Japan, Tel: +81 133 23 2484.

*Corresponding author: nakazawa@hoku-iryo-u.ac.jp

doi: 10.15414/jmbfs.2016/17.6.3.961-965

ARTICLE INFO

Received 14. 1. 2016
Revised 24. 10. 2016
Accepted 25. 10. 2016
Published 1. 12. 2016

Regular article

OPEN ACCESS

ABSTRACT

This study to investigate the effect of roselle calyx extract on gingipain activity, production of inflammatory cytokines, and oral bacterial morphology. The inhibitory effect of roselle calyx extract (RCE) on Arg- (Rgp) and Lys-gingipain (Kgp) was evaluated using synthetic substrate colorimetric assay with a 96-well microtiter plate. RCE with various concentrations was tested for determination of interleukin (IL)-6 and IL-8 produced from KB cells stimulated with heat-inactivated *P. gingivalis.* The production of IL-6 and IL-8 was quantified using an ELISA kit. The morphological alterations in cells of *S. mutans* and *P. gingivalis* after treatment with RCE were studied using scanning electron microscopy (SEM). RCE could inhibit both Rgp (90%) and Kgp (70%) activities significantly at concentrations lower than the minimum inhibitory concentration (MIC) (2.2 mg/mL). Furthermore, the production of IL-6 and IL-8 from KB cells stimulated with *P. gingivalis* was significantly inhibited by RCE at 6 h after exposure, in a dose-dependent manner. After treatment with RCE, the morphological alterations were observed in cells of *S. mutans* and *P. gingivalis* by using SEM. The ability of RCE to inhibit gingipain activity and production of inflammatory cytokines indicates that RCE could be considered for prevention and clinical treatment for periodontitis.

Keywords: Roselle calyx extract, Rgp, Kgp, *Porphyromonas gingivalis*, IL-6, IL-8

INTRODUCTION

Periodontitis is an oral infectious disease caused by bacteria or groups of bacteria. The bacteria accumulate on the surface of the tooth and cause the destruction of periodontal connective tissue and alveolar bone, resulting in tooth loss (**Haake *et al*., 2006; Darveau, 2010**). It was reported that a group of bacteria, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, and *Fusobacterium nucleatum* may contribute to periodontal diseases. Among these, *Porphyromonas gingivalis* (*P. gingivalis*), a Gram-negative, anaerobic, non-motile rod, is strongly associated with adult periodontitis (**Slots *et al*., 1994**). This species is significantly more abundant in diseased sites than in healthy sites (**Slots *et al*., 1999; Haffajee *et al*.,1994**). The presence of *P. gingivalis* is indicative of disease progression risk and reduced levels correlates with successful clinical treatment (**Haffajee *et al*.,1994**).

P. gingivalis possesses many virulence factors, including gingipains. Gingipains are trypsin-like cysteine proteinases classified into two groups, the arginine-specific cysteine protease (Rgp) and the lysine-specific cysteine protease (Kgp), based on substrate specificity. Rgp activates the blood coagulation system, associated with gingival crevicular fluid production, and initiates the inflammation process (**Imamura, 2003**). Kgp contributes to a tendency toward gingival bleeding (**Imamura, 2003; Potempa *et al.*, 2000**). The gingipains have been isolated from culture supernatants, vesicle membrane fractions, and cell extracts (**Chen *et al*., 2001; Yamanaka *et al*., 2007**).

Epithelial cells, which line mucosal surfaces and provide an important mechanical barrier, play a communication role for the host as microorganism sensors and signal providers. These cells can activate mucosal inflammatory and immune responses(**Kagnoff *et al*., 1997**). It has been reported that *P. gingivalis* can bind to and invade human oral epithelial cells (**Duncan *et al*., 1993**). An interaction between bacteria and epithelial cells is required for periodontal inflammation (**Yumoto *et al*., 1999**). Cytokines, small soluble proteins produced by a cell, play an important role in many biological activities such as inflammation (**Okada *et al*., 1998**). Inflammatory cytokines, like interleukin-(IL) 6 and IL-8, are induced during the inflammatory process. Some reports showed the involvement of IL-6 and IL-8 in the pathogenesis of tissue destruction in periodontitis (**Yumoto *et al*., 1999; Okada *et al*., 1998; Kang *et al*., 2011**). Since gingipains, IL-6, and IL-8 are involved in the pathogenesis of periodontitis, these components could be effective targets for prevention/control of periodontitis.

Roselle calyx extract (RCE), the extract from *Hibiscus sabdariffa* L. (Family Malvaceae) grows in tropic and subtropic areas. Roselle calyx has medicinal properties and has been reported to contain alkaloids, saponins, and flavonoids such as gossypetine, hibiscetin, and sabdaretine (**Hirunpanich *et al*., 2005; Olaleye *et al*., 2007**). It also contains hibiscus acid, hydroxybenzoic acids, flavonols, anthocyanins, and other polyphenolic compounds (**Rodrigues *et al*., 2011**). RCE has been considered to have antihypertensive (**Arellano *et al*., 2004**) hepatoprotective (**Ali *et al*., 2003**), antihyperlipidemic (**Hirunpanich *et al*., 2006**), antioxidant (**Yang *et al*., 2012**), anticancer (**Tsai *et al*., 2014**), and antimicrobial (**Olaleye *et al*., 2007; Nwaiwu *et al*., 2011**) properties. It is possible that the use of plant extracts with the ability to inhibit gingipains activity and cytokine production is an alternative strategy for periodontal therapy. Our previous study showed that RCE has antibacterial activity and inhibits biofilm formation. In the present study, we investigate RCE's ability to inhibit gingipains activities and cytokine production stimulated by *P. gingivalis*, and its effect on bacterial morphology.

MATERIAL AND METHODS

Preparation of the RCE

We soaked 16 g powder of dried roselle calyx in 160 mL ethyl alcohol (Wako Pure Chemical Industries Ltd) with shaking for 24 h at room temperature. After centrifugation, the extract was lyophilized. Then the extract was dissolved in phosphate-buffered saline (PBS) and the pH was adjusted to 7.0. The extract was aseptically filtered through a disposable membrane filter unit with a 0.45-µm pore size. The extract was stored at - 20°C for further use.

Determination of Rgp and Kgp activity

The inhibitory effect on Rgp and Kgp of RCE at different concentrations was evaluated by using synthetic substrate colorimetric assay (**Yamanaka *et al.*, 2007; Nakatsuka *et al.*, 2014**). Benzoyl-arginine-*p*-nitroanilide (Sigma-Aldrich) and tosyl-glycine-proline-lysine-*p*-nitroanilide (Sigma-Aldrich), in 100 µl of 0.1 M Tris-HCl (pH 8.0) containing 1 mM dithiothreitol were used as substrates (final concentration 0.5 mM) for Rgp and Kgp, respectively. The substrates were dispensed into the wells of a 96-well microtiter plate. A bacterial cell suspension (50 µl) of *P. gingivalis*, 1 x 10^8 cells/ml in PBS and RCEs at different concentrations, were added to the substrate and incubated at 37°C for 50 min. The optical density, OD, of each well was measured by microtiter plate reader at a wavelength of 405 nm (A_{405}). Relative enzymatic activity was determined as follows: [(A_{405} with bacterial cells and RCE – A_{405} of control) / (A_{405} with bacterial cells – A_{405} of control)] x 100. Degradation obtained in the absence of the RCE was given a value of 100%.

Determination of IL-6 and IL-8 production

KB cells (a human mouth epithelial cell line) were grown in a 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (100 µg/ml). After washing twice with PBS, the cells were detached from the cultured plate and then diluted to obtain 1 x 10^5/ml. A cell suspension (100 µl) was cultured in a 96-well plate and incubated to about 90% - 100% confluence. Bacterial cells of *P. gingivalis* (1 x 10^8 cfu) were suspended in serum-free DMEM and heat-inactivated at 80°C for 30 min before infection of KB cells. RCE in various concentrations and 60 µl of the bacterial suspension were incubated with KB cells at a multiplicity of infection (MOI) of 200 at 37°C in 5% CO_2. After 6 h, the supernatants were collected and stored at -20°C for cytokine assays. The levels of cytokine in the culture supernatant were determined by ELISA kit (Thermo, USA) according to the manufacturer's instructions. Briefly, the lyophilized standard or RCE was placed on a 96-well strip plate precoated with anti-human cytokine. Afterward, biotinylated antibody reagent was added to each well, followed by streptavidin-horseradish peroxidase solution. Tetramethylbenzidine (TMB) substrate was added and the reaction was stopped by adding stop solution containing 0.16 M sulfuric acid. The absorbance was measured on a plate reader at 450 nm.

Examination of bacterial morphology with Scanning Electron Microscopy

A scanning electron microscopy (SEM) was performed to examine the changes of bacterial morphology after treatment with RCE. In this study, *S. mutans* ingbritt and *P. gingivalis* ATCC 33277^T were used. Bacterial suspensions were adjusted to reach OD 1.0 at 600 nm in PBS after overnight culture and washing. These suspensions were layered on cell culture coverslip disks (Thermo Scientific, Rochester, NY, USA) by soaking for 2 h in anaerobic conditions. The layered disks were washed three times and then treated either with RCE at three times minimum inhibitory concentration (MIC) (21.6 mg/mL) or with PBS for 1 h or 2 h at room temperature. After being washed, they were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h. The resultant glutaraldehyde-treated layered disks were then washed and dehydrated in ethanol (50, 70, 80, and 90% successively), each for 10 min, and finally three times with 100% ethanol for 20 min each. They were dried by the CO_2 critical-point drying technique (HCP-2, Hitachi, Tokyo, Japan), coated with gold in vacuum (Eiko, IB-3 ion coater, Japan) and examined using SEM (Hitachi S-3500N, Japan).

Statistical analysis

Statistical analysis was performed using SPSS 21 software. Results were obtained in triplicate and were expressed as mean ± standard deviation (SD). The significance of the differences between groups was determined using independent *t*-test with a value of $p < 0.05–0.01$; the effect of RCE on *P. gingivalis*-induced IL-8 expression in KB cells was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

RESULTS

Effect on Rgp and Kgp activity

The effect of RCE on Rgp and Kgp activity was evaluated by colorimetric assay using synthetic substrates (Figure 1). The present study showed that RCE exhibited inhibitory effect on both Rgp and Kgp activity in a dose-dependent manner. RCE reduced the activity of Rgp about 60% at 0.6 mg/mL, 80% at 1.1 mg/mL, and 90% at 2.2 mg/mL. The reduction in Kgp activity was about 50% at 0.6 mg/mL, 60% at 1.1 mg/mL, and 70% at 2.2 mg/mL. The differences in gingipains activity between the untreated and treated groups were statistically significant ($p<0.05$).

Effect on IL-6 and IL-8 production

We evaluated the effect of RCE at various concentrations on the production of IL-6 and IL-8 by KB cells stimulated with the heat-inactivated *P. gingivalis*. In Figure 2, the levels of IL-6 (Figure 2A) and IL-8 (Figure 2B) produced by KB cells were significantly reduced by treatment with RCE in a dose-dependent manner ($p < 0.01$) with similar reduction patterns.

Effect on bacterial morphology

Figure 3 shows the morphological alteration of *S. mutans* and *P. gingivalis* after treatment with RCE for 1 h (3E and G) and 2 h (3F and H) compared to the structure of the controls (3A, B, C, and D). Bacterial cells of *S. mutans* and *P. gingivalis* in the control group had regular shapes and smooth surfaces. After treatment with RCE, some *S. mutans* cells showed irregular changes such as enlargement and clumping of cells. In *P. gingivalis*, treatment with RCE caused aggregation and distortion of cells. These morphological alterations increased after 2-h treatment.

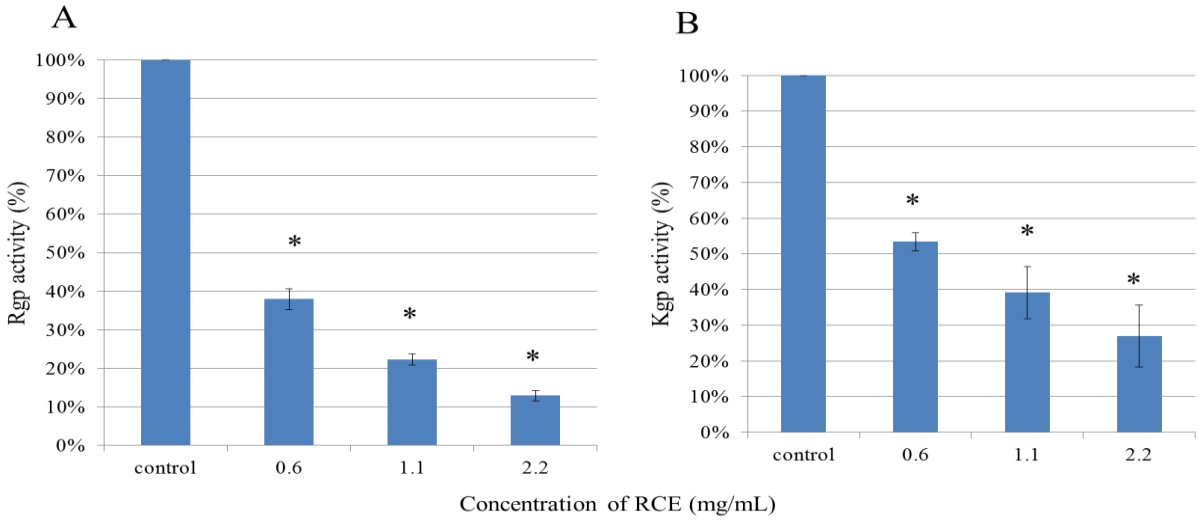

Figure 1 Effect of roselle calyx extract on Rgp (A) and Kgp (B) activity of *P. gingivalis*. The data was expressed with the mean ± SD in triplicate experiment. * $p < 0.05$: significantly different from the control. Control: absence of RCE; Rgp: Arg-gingipain; Kgp: Lys-gingipain; RCE: roselle calyx extract.

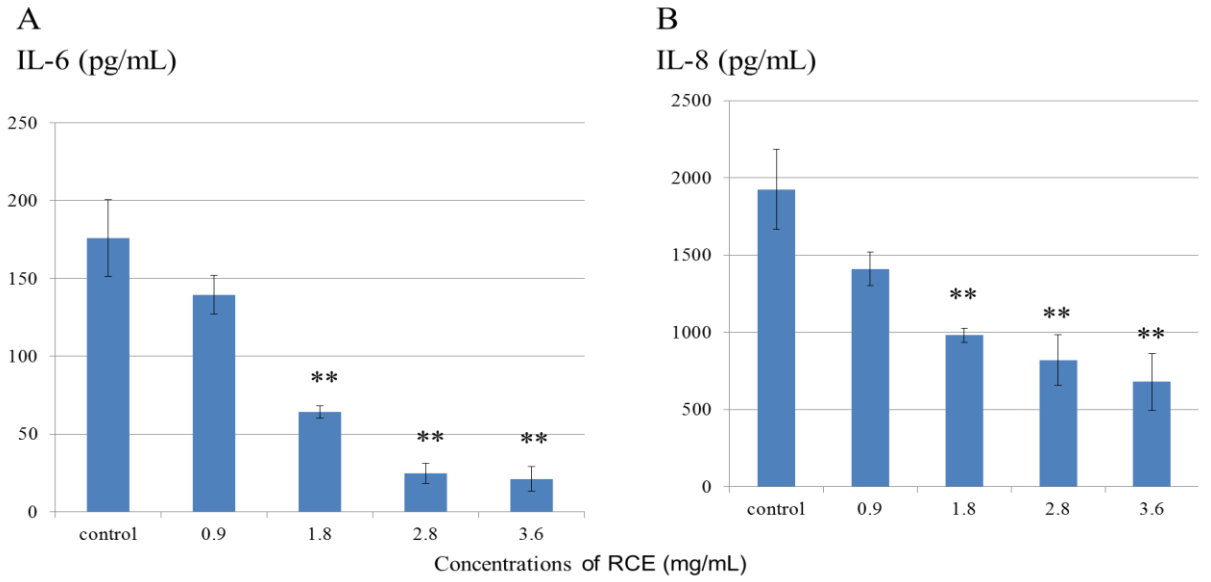

Figure 2 Effect of RCE on *P. gingivalis*-induced IL-6 (A) and IL-8 (B) expression in KB cells. Control: PBS, *P. gingivalis* was used at MOI 200. ** $p \leq 0.01$: significantly different from the control. The data is expressed with the mean ± SD in triplicate experiment.

DISCUSSION

Many bacterial species have been identified in periodontal pockets of patients; among those *P. gingivalis* is frequently isolated from the lesions of chronic periodontitis. It was reported that *P. gingivalis* produced major proteinases, Arg-specific gingipain (Rgp) and Lys-specific gingipain (Kgp), as virulence factors (**Kataoka *et al*., 2014**). Gingipains play a key role in the pathogenic function of *P. gingivalis*. They have the ability to degrade a number of host proteins significant for bacterial growth and metabolism, and are responsible for dysregulation of host defensive inflammatory reaction and failure of the host to eliminate bacteria (**Olsen *et al*., 2014**).

Gingipains are located on cell surfaces and in culture supernatants, and are related to extracellular vesicles (**Chen *et al*., 2001**). The gingipain-loaded outer membrane vesicles may contribute to tissue destruction in periodontal diseases by serving as a vehicle for the antigens and active proteases (**Nakao *et al*., 2014**). When separate proteinases with arginine and lysine specificity were isolated from a high molecular mass fraction of the *P. gingivalis* culture fluid, the arginine- and lysine-gingipains were found to contain a hemagglutinin complex. This hemagglutinin may be important in the uptake of hemin via hemagglutination and subsequent hemolysis of erythrocytes (**Pike *et al*., 1994**). Arginine-specific cysteine proteinase from the culture supernatant of *P. gingivalis* has the ability to disrupt the functions of polymorphonuclear leukocytes. Further, the enzyme was suggested as a major virulence factor from *P. gingivalis* in the progression of periodontal diseases due to direct destruction of periodontal tissue components and the disruption of the normal host defense mechanism (**Kadowaki *et al*., 1994**). The culture supernatant from *P. gingivalis* also induces the disruption of the adhesion and proliferation activities in the endothelial cells; Rgp and Kgp are responsible for these activities (**Baba *et al*., 2002**). It is clear that while gingipains are important for the survival of bacteria, they are also pathogenic to the host. Therefore, both Rgp and Kgp activities should be potential therapeutic targets (**Olsen *et al*., 2014**).

Results in our study indicate that RCE inhibited the activity of Rgp and Kgp at a sub-MIC concentration (Figure 1). Our previous study reported that the MIC of *P. gingivalis* is 7.2 mg/mL (**Sulistyani *et al*., 2016**). The concentration of RCE used in this study was sub-MIC, therefore the decrease of Rgp and Kgp activity is not because of inhibition of bacterial growth, but because of inactivity of Rgp and Kgp. It was reported that RCE contains polyphenolic compounds (**Rodrigues *et al*., 2011**). Previous study showed that cranberry polyphenol was found to inhibit Arg- and Lys-gingipain activities in a dose-dependent manner (**Yamanaka *et al*., 2007**). Polyphenols from *Myrothamnus flabellifolia* Welw (MF) exhibit inhibition of *P. gingivalis* adhesion to epithelial cells, with strong influence against Arg-gingipain. In *P. gingivalis*, the gingipain proteases are suitable targets for polyphenol compounds. Proanthocyanidins react with bacterial surface proteins, leading to an unspecific cross-linking or denaturing effects of adhesion proteins and resulting in inhibition of Rgp (**Lőhr *et al*., 2011**). From these data, we assume that the inhibition effect of RCE on Rgp and Kgp may be due to polyphenol compounds. However, MF exhibits high toxicity, which may limit its clinical use, whereas RCE is safe to use.

Periodontopathogens can promote host inflammatory response by secretion of inflammatory mediators. The association of inflammatory mediators and local tissue destruction in periodontitis has been reported (**Okada *et al*., 1998; Birkedal, 1993; Bodet *et al*., 2007**). IL-6 is a pro-inflammatory cytokine that has been related to the pathogenesis of periodontal disease. IL-6 expression is increased at sites of periodontal inflammation. It has been suggested as a marker for periodontal disease activity (**Irwin *et al*., 1998**) and is involved in bone resorption (**Nakashima *et al*., 2000**). It was reported that IL-8, expressed in epithelial cells and macrophages, was observed in gingival inflammation. IL-8 may play an important role in the pathogenesis of periodontitis due to its pro-inflammatory and neutrophil chemotactic properties (**Okada *et al*., 1998; Kim *et al*., 2006**). The neutrophils are the first line of defense against periodontopathic bacteria. Continuous and excessive IL-8-mediated chemotactic and activation effects on neutrophils may contribute to local destruction of periodontal tissues (**Okada *et al*., 1998**).

In the present study, we analyzed whether KB cells stimulated by the heat-inactivated *P. gingivalis* for 6 h could produce IL-6 and IL-8. Previous study also reported that KB cells showed cytokine responses after infection with *P. gingivalis*, strongly comparable to that induced by *E. coli* in vitro (**Sandros *et al*., 2000**). The ability of epithelial cells to provoke the cytokine response was related to their adhesive and invasive capacity. In a comparative experiment using a primary culture of pocket epithelium and KB cells, the result revealed that cytokine responses after *P. gingivalis* and *E. coli* infection were similar.[36] In addition, in vivo study showed that *P. gingivalis* was more potent than *A. actinomycetemcomitans* in inducing proinflammatory cytokines expression. Animals infected with *S. gordonii*, considered a non-pathogenic bacteria, also induced IL-1ß and TNFα but not IL-6 (**Kesavalu *et al*., 2002**).

Our study exhibited that the production of IL-8 (1926 pg/mL) is higher than that of IL-6 (176 pg/mL). Our result is in accordance with previous studies; **Yumoto *et al*. (1999)** reported that the level of IL-8 production (approx. 2300 pg/mL) by KB cells stimulated with *E. corrodens* for 8 h is higher than the level of IL-6 production (approx. 98 pg/mL). The level of IL-6 increased 0.5 h after stimulation; the concentration increased constantly over time and it increased significantly within 4 h. In contrast, the level of IL-8 increased 4 h after stimulation, and increased aggressively from that point. The production of IL-8, higher than that of IL-6, was also shown in KB cells infected by *F. Nucleatum* (**Kang *et al*., 2011**).

The level of IL-6 and IL-8 response in KB cells induced by heat-inactivated *P. gingivalis* was reduced after treatment with RCE at all concentrations used in this study. No cytotoxic effects were detected by WST-1 assay in KB cells treated by RCE in those concentrations for 6 h (data not shown). It is indicated that the reduction of IL-6 and IL-8 production in KB cells treated by RCE is not associated with cell toxicity. In addition, treatment of uninfected KB cells with RCE did not affect cytokine responses (data not shown). It is shown that treatment by RCE does not affect KB cell production of IL-6 and IL-8.

It was reported that RCE contains polyphenols, with flavonoids being the largest of the dietary phenolics group (**Kim *et al*., 2006; Carretero *et al*., 2008; Medina *et al*., 2009**). Flavonoids have anti-inflammatory effects. Previous study showed that the non-dialyzable material (NDM) cranberry fraction, rich in polyphenolic compounds, could inhibit inflammatory mediator production (**Bodet *et al*., 2007**). The mechanism of inhibition may be via a down-regulation of the activator protein-1 (AP-1) activity. AP-1 and nuclear factor-κB (NF- κB) are known pro-inflammatory transcription factors that induce the production of cytokines and other pro-inflammatory molecules (**Verri *et al*., 2012**). AP-1 and NF- κB co-operatively regulate the synthesis of IL-6, IL-8, and PGE_2 in the gingival

connective tissue of patients with periodontitis (**Bodet *et al*., 2007; Kida *et al*., 2005**). Epigallocatechin gallate (EGCG), the major polyphenol component of green tea, inhibits IL-1ß-induced IL-8 production of nasal fibroblast and A549 epithelial cells. The activity might be associated with an intervening reactive oxygen species (ROS) pathway (**Kim *et al*., 2006**). Because the main compound of RCE is a flavonoid, we suggested that the flavonoid is involved in the inhibitory effect on IL-6 and IL-8 in KB cells induced by heat-inactivated *P. gingivalis*. The use of flavonoids as an anti-inflammatory may represent a better pharmacological approach compared to current therapies because flavonoids do not act by a single mechanism and present a better side effect profile, reducing such effects as gastrointestinal and renal lesions (**Kida *et al*., 2005**).

The morphologic alteration of oral bacterial species by treatment with RCE has not been reported previously. In this study, we investigated the effect of RCE on *S. mutans* and *P. gingivalis* using SEM. The layered disks of bacteria were treated with RCE at three times MIC for 1 h or 2 h under appropriate conditions. We used RCE at this concentration because our purpose was to observe morphologic alterations on the bacteria rather than a reduction in bacteria. In addition, using a concentration of RCE equal to MBC may have resulted in bacteria detaching from coverslip disks when dead bacteria were processed and removed. The SEM images illustrate the detrimental effects of RCE on the bacteria. *S. mutans* and *P. gingivalis* in the control group had regular shape and smooth surfaces. After 1 h of treatment, some cells of *S. mutans* showed irregular shape or clumping. After 2 h, some bacteria showed variation in size, some cells were expansive, and the clumping of cells was more evident than at 1 h of treatment. Treatment by RCE on *P. gingivalis* for 1 h caused aggregation and irregular cell outlines. These patterns were increased on cells treated by RCE for 2 h.

It has been reported that RCE contains phenolic compounds, such as flavonoid. The target site of flavonoid might be on membrane cell walls (**Cowan, 1999**). Our SEM result was in accordance with previous study and showed catechin, the polyphenol compound, caused leakage of intramembranous materials, strong aggregation, and bacterial cell pointing to the likely possibility of membrane fusion. It is well established that membrane fusion by polyethylene glycol, calcium ion, or virions has consistently resulted in the leakage of intramembranous materials and aggregation (**Ikigai *et al*., 1993**). SEM analysis of oral microorganisms treated with tea polyphenol showed major structural cell surface changes and irregular forms, with aggregates among cells (**Cho *et al*., 2010**). It was reported that damage to bacterial cells might manifest in a few ways, one of which was loss of membrane integrity resulting in leakage of essential intracellular constituents such as inorganic phosphate and proteins. The target of phenols was membrane integrity and leakage (**Denyer *et al*., 1998**). From the SEM observations, it seems that RCE binds to the cell membrane, penetrates into the phospholipid bilayer, and disrupts the membrane integrity, resulting in membrane leakage and cell aggregation. We suggested after aggregation, the bacteria will lose integrity and disintegrate, resulting in death. However, the mechanisms of RCE on oral bacteria remains unclear, therefore experiments need to be developed.

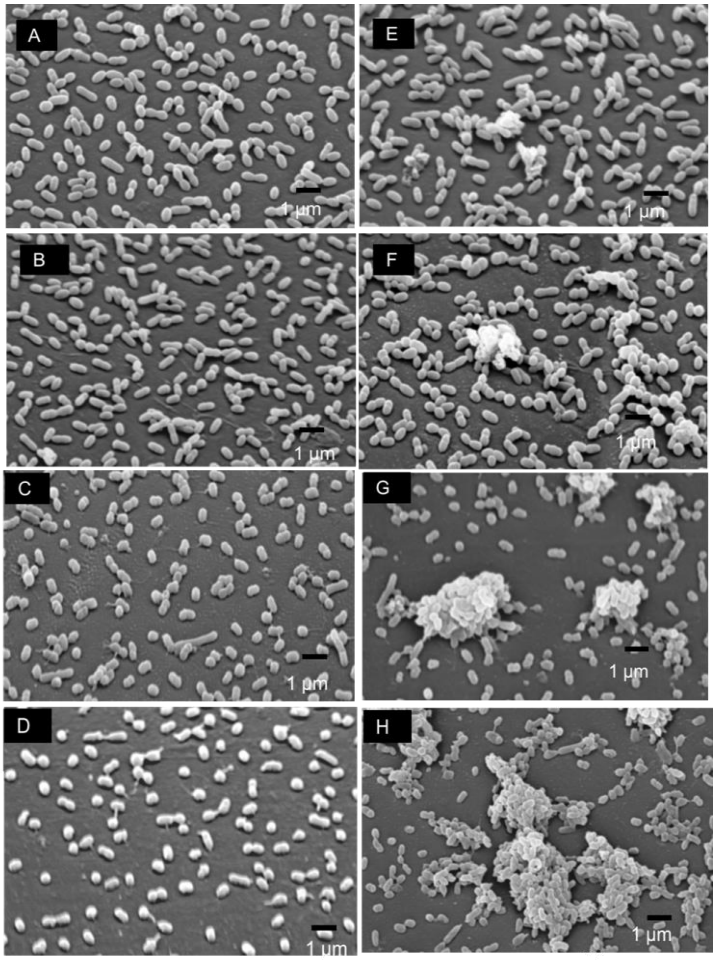

Figure 3 Scanning electron microscope *S. mutans* (A, B, E and F) and *P. gingivalis* (C, D, G and H). A and C: PBS 1 h; B and D: PBS 2 h; E and G: treatment with RCE for 1 h; D and H: treatment with RCE for 2 h.

CONCLUSION

Based on our results, RCE could inhibit the activity of Rgp and Kgp, the major proteases of *P. gingivalis*. Moreover, RCE inhibited IL-6 and IL-8 production in KB cells stimulated with the heat-inactivated *P. gingivalis*. From the SEM observations, we suggested that RCE binds to the cell membrane, resulting in membrane leakage and cell aggregation. These data suggest that RCE might have beneficial effect on prevention and clinical treatment for periodontitis.

Acknowledgments: We thank Dr. Yuko Nakatsuka and Akio Maruyama (Health Sciences University of Hokkaido) for technical support. Part of this work was supported by scholarship from Futoku Foundation and Hokkaido Gas Co. Ltd.

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