Chewing Gum Containing Citric Acid Reduces the Burden of Periodontal Pathogens

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Abstract: Objective: In a preliminary study, we noted that citric acid has the potency to induce human beta defensins (hBD-2) in salivary secretions. As hBDs are thought to ameliorate periodontal disease by suppressing key periodontal pathogens we developed a citric acid-containing chewing gum (CA-gum) to test this hypothesis. Here we report, the effect of CA-gum on three major periodontal pathogensPrevotella intermedia (Pi), Tannerella forsythia (Tf), Treponema denticola (Td) and Fusobacterium species (Fb).

Methods: A randomized, double-blind, placebo-controlled trial with 61 individuals, was conducted over 14 days. Each subject was allowed to chew CA-gum (31 subjects) or placebo gum (30 subjects) at irregular intervals, but was instructed to eat at least three pieces of gum a day. None had active caries, symptoms of gingivitis or periodontal disease, smoking history, or concurrent medication. To determine the levels of oral carriage of the putative pathogens, saliva was collected from each subject by paraffin chewing in a standard manner and, the number of total cultivative microorganisms (CFUs) determined. Five key periodontal pathogens, Pg, Pi, Tf, Td and Fb present in saliva were determined quantitatively using real-time polymerase chain reaction (PCR) (BML Inc., Saitama, JAPAN).

Results: Compared with the placebo chewing gum group the CA-gum group had significantly lower levels ofPrevotella intermedia, Tannerella forsythia, and Fusobacterium spp. in their saliva. (Wilcoxon t-test; p<0.01). However, chewing of either type of gum did not affect the salivary carriage of total cultivative bacteria (Wilcoxon t-test; p>0.05).

Conclusion: Consumption of citric acid-laced chewing gum reduces significantly the burden of periodontal pathogens in saliva.

Keywords: Saliva, beta defensin, periodontal bacteria, citric acid.

1. INTRODUCTION

Periodontal disease is a common oral disease characterized by a chronic inflammatory process accompanied by destruction of surrounding connective tissue and alveolar bone, and sometimes loss of teeth [1]. The primary causative agents of periodontal disease are particular gram-negative anaerobic bacteria that accumulate in the gingival sulcus.

Bacterial colonization triggers both an innate immunity and host response and acquired immunity. The numerous enzymes and antimicrobial factors present in the saliva and crevicular fluid aid in the neutralization of bacterial components of supragingival and subgingival microbial biofilms. These include lysozyme, lactoferrin, peroxidase, bradykinin, thrombin, fibrinogen, complements, antibodies, and neutrophil-derived components and antibacterial peptides [2-4].

The human beta defensins (hBDs), in particular, are small, cationic, cysteine-rich peptides with potent antimicrobial activity against both gram-positive and –negative bacteria, fungi, and viruses [5], and are produced by epithelial cells of the skin, many lining mucosa, such as intestinal, airway and oral and/or gingival tissues. Among the hBDs, a number of in vitro studies have shown the inducibility of hBD-2 in human keratinocytes exposed to proinflammatory cytokines and components of microorganisms, implying its more specialized role in the innate defense compared with hBD-1, which is less active in disease state and less effective in killing the several microorganisms [6,7].

The expressions of hBDs, particularly hBD2, in vivo, were reported to play a role in protection from periodontal disease. It is suggested that hBD-1 and -2 were frequently
expressed in the granular and spinous layers of gingival epithelia and their expression might be associated with periodontal health and disease [8]. In addition, the fact that high levels of hBD2 and 3-mRNA expressed in healthy tissues, as compared with tissues in the status of periodontitis, suggest an important protective role for hBDs in the host immune response to infection by periodontal pathogens [9].

Dunsche et al. have shown that hBDs were expressed widely in oral tissues, not only in epithelial cells but also in salivary glands, such as parotid glands, submandibular glands, small labial glands and sublingual glands [10]. On the other, Fehlbaum et al. has shown that small amino acids including L-isoleucine and several of its analogs can specifically induce epithelial β-defensin expression [11]. Hence, these findings, taken together, suggested the possibility that the stimuli on the oral tissues particularly salivary glands, by amino acids or acids, could induce the hBD-2 together with salivary flow, which will be expected to be an aid to control oral diseases like periodontal disease.

Both the chewing and acids are beneficial materials to induction of salivary flow, and the two have been combined in an acid-containing chewing gum under the hypothesis that a kind of acid could stimulate the salivary flow together with an increase secretion of hBD-2 which will be expected to reduce the periodontal pathogens. Hence, in the present study, we examined i) the acid potent to induce both salivary flow and hBD-2, and ii) the effects of dairy gum chewing containing acid, on the oral flora and pathogens.

2. MATERIALS AND METHODS

2.1. Participants and Saliva Collection

Six healthy adult volunteers (3 males: 3 females; mean age 37.8 ± 3.5) took part in the assay, and were able to provide informed consent. To be included, volunteers had to be non-smokers and have no significant oral, dental or systemic diseases; not taking any medication.

Each volunteer rinsed the mouth with water at least 30 minutes before the saliva collection. Then the unstimulated saliva was collected in advance of the stimulated saliva collection as a negative control.

The stimulated saliva was induced by the load of 50 miligrams of powder or 1.0 mL of 5% acid solutions which were commonly used for foods, such as, citric acid, DL-malic acid, boletic acid, glutamic acid, cinnamic acid, ascorbic acid, succinic acid, tartaric acid, adipic acid, lactic acid (solution) and acetic acid (solution). Stimulated saliva was collected into a container on ice at intervals of 0-1, 1-2, 2-3 and 5-6 min after the load of each acid to determine the volume of saliva secreted and the concentration of salivary hBD-2.

The data were analyzed by one way analysis of variance (ANOVA) and Tukey’s multiple range test at 1% level.

2.2. Determination of hBD-2 Levels in Saliva

The concentration of salivary hBD-2 was determined by the use of enzyme-linked immunosorbent assay (ELISA). Each saliva sample was diluted appropriately by phosphate-buffered saline supplemented with 10% calf serum. Anti-hBD-2 rabbit serum was purchased from Peptide Institute Inc. (Minoh-city, Osaka Japan), and EZ-Link NHS-LC-Biotin purchased from PIERCE Biotechnology Inc., (Rockford, IL, USA) were used for biotin-label. Streptavidin and horseradish peroxidase (HRP) conjugate were purchased from Funakoshi Co. Ltd. (Tokyo, JAPAN). Synthesized hBD-2 (BIO PHARM LIMITED, Shizuoka, JAPAN) was used to standardize the concentration of salivary hBD-2.

The data were analyzed by one-way analysis of variance (ANOVA) and Tukey’s multiple range test at 1% level.

2.3. Effects of Chewing Gum Containing Citric Acid (CA-gum) and Placebo Chewing Gum (Placebo Gum) on Oral Flora and Pathogens

A total of 61 healthy subjects was divided into two groups. None of subjects had active caries, symptoms of gingivitis or periodontal disease, smoking habit, or any medication. The compositions of CA-gum and placebo gum are summarized in Table 1. The first group comprising 31 subjects (mean age 19.2 ± 0.4) ate placebo gum, and the second group (30 subjects, mean age 20.4 ± 0.8) ate CA-gum for a period of two weeks. Each subject was allowed to eat the chewing gum at spontaneous intervals daily, but instructed to eat at least 3 pieces a day. One piece of CA-gum (3.2g) contains 200mg citric acid.

### Table 1. Composition of Placebo Gum and CA-Gum

<table>
<thead>
<tr>
<th></th>
<th>Placebo Gum</th>
<th>CA-Gum</th>
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</thead>
<tbody>
<tr>
<td>maltitol</td>
<td>61.08</td>
<td>60.15</td>
</tr>
<tr>
<td>gum base</td>
<td>22.1</td>
<td>22</td>
</tr>
<tr>
<td>hydrogenated starch hydrolysate</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Citric acid anhydrous</td>
<td>0</td>
<td>6.25</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.71</td>
<td>0.5</td>
</tr>
<tr>
<td>Acesulfame potassium</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Stevia</td>
<td>0.05</td>
<td>0.05</td>
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<tr>
<td>Total</td>
<td>100 (%)</td>
<td>100 (%)</td>
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Before and after eating each variety of chewing gum, the levels of oral carriage of microorganisms were determined, as follows. The saliva collections were carried out at least 2 h after lunch. The subjects were asked to chew a piece of paraffin for 10 min, and the saliva produced was expectorated into a plastic container. The number of total cultivative microorganisms was determined with conventional viable counts. Periodontal pathogens released in saliva, such as Porphyromonas gingivalis (P.g.), Prevotella intermedia (P.i.), Tannerella forsythia (T.f.), Treponema denticola (T.d), and Fusobacterium spp. (Fuso.) were determined quantitatively using real-time polymerase chain reaction (PCR) by BML Inc. (Saitama, JAPAN). Initially, the effects of CA-gum and placebo gum on salivary flow and salivary hBD2 were examined one week before the start of gum-chewing periods with the same divided groups. Each volunteer rinsed the mouth with water at least...
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30 minutes before saliva collection. Then the unstimulated saliva was collected in advance the collection of gum-stimulated saliva as a negative control. Then the subjects were asked to chew a piece of CA-gum or placebo gum for 1 min, and the saliva produced was expectorated into a plastic container on ice to determine the volume of saliva secreted and the concentration of salivary hBD-2 by ELISA.

Both the subjects and investigators were unaware of which chewing gum was the CA gum or placebo gum throughout the study. The use of dairy products like Yoghurt or other chewing gum was forbidden for one week prior to and throughout the intervention. The data were analyzed by Wilcoxon test at 1 and 5% levels.

2.4. Ethical Approval and Informed Consent

The protocols of saliva sampling and cohort study using chewing gum have been approved by Ethical Authorities at Hiroshima University (22th January 2005). Informed consent was obtained from all the participants after the explanation of both the merits and demerits of the protocol and samples used for the experiment. Because the CA-gum included a high concentration of citric acid, which is known as a causative factor of enamel erosion, we examined the occurrence of dental erosion or troubles in oral health of each participant twice a week, during the periods of cohort tests, to prevent the erosions or other side effects.

All participants were provided the instruction not to take acid flavor foods, beverages or supplements, like chewing gum, candy, pickled Japanese apricot (pickled ume), yogurt and other lactic acid drinks, and/or foods containing lactic acid, during the period of experiment.

3. RESULTS

3.1. Induction of hBD2 in Saliva by Various Acids

The amount of hBD2 in saliva rapidly increased with salivary flow after the load of acid (Fig. 1), implying the possibility that some acids were able to induce hBD2 in saliva as expected. Accordingly, which acid is most potent to induce saliva and/or salivary hBD2 was examined. As shown in Fig. (2-a), tartaric acid was most potent to induce saliva, followed by lactic acid, glutamic acid, cinnamic acid, and reduced in the order of boletic acid, malic acid, ascorbic acid, succinic acid, citric acid, and adipic acid and acetic acid showed the lowest (ANOVA & Tukey’s multiple range test; p<0.01). The potential of each acid to induce salivary hBD2 was quite different from that to induce saliva (Fig. 2). Citric acid showed the highest potential to induce hBD2 in saliva, followed by malic acid and boletic acid, and tartaric, succinic, acetic, adipic, ascorbic, glutamic and cinnamic acid showed the lowest (ANOVA & Tukey’s multiple range test; p<0.01), which were not significantly different from induction of hBD2 by water or hBD2 in unstimulated saliva. Thus we have chosen the citric acid to induce the salivary hBD2 hereafter.

3.2. Effects of Chewing Gum Containing Citric Acid (CA-gum) and Placebo Chewing Gum (Placebo Gum) on Oral Bacteria and Periodontal Pathogens

As shown in Fig. (3), there were no significant differences observed in either the volume of unstimulated saliva or the concentration of salivary hBD2 between group 1 and group 2. The volume of saliva and the concentration of hBD2 in saliva were significantly increased by chewing either type of gum. The increase in the volume of saliva was significantly higher with the group 2 which ate CA-gum, than those with group 1 (Wilcoxon t-test, p<0.01). Similarly, the concentration of salivary hBD2 was significantly higher with group 2 which ate CA-gum, than with group 1 (Wilcoxon t-test, p<0.01).

The chewing of either type of gum did not affect the oral carriage of total cultivative bacteria (Wilcoxon t-test; p>0.05).
Fig. (2). The potential of acids to induce saliva (○) and concentration of induced salivary hBD2 (●).

Placebo gum did not significantly reduce the P.g., whereas CA-gum tended to reduce the P.g., but not significantly (Wilcoxon t-test; p>0.05). CA-gum significantly reduced the oral carriage of P.i. (Fig. 4; p<0.01), but this was not observed with placebo gum (Fig. 4; Wilcoxon t-test; p>0.05). The oral carriage of T.f. was significantly reduced by CA gum (Fig. 5; p<0.01), and placebo gum also tended to reduce the T.f., but not significantly (Wilcoxon t-test; p>0.05). Neither gum had significant effects on the oral carriage of T.d., but against Fus.o., CA gum reduced significantly (Fig. 6; Wilcoxon t-test; p<0.01).

During the periods of cohort tests, we checked the oral health of each participant and there were no symptom of erosion or side effects caused by routine chewing of CA-gum.

4. DISCUSSION

4.1. Induction of hBD2 in Saliva by Various Acids

In the oral cavity, antimicrobial peptides secreted in saliva or crevicular fluids, such as defensin, histatin, and cathelicidin play a pivotal role as a first-line defense against a succession of invading bacteria [12]. HBDs exert their bactericidal activity by acting on the bacterial membrane, and epithelial cells of the oral mucosal barrier upregulate hBD-2 and hBD-3 upon stimulation by bacterial components or inflammatory mediators [6,13]. Thus the induction of saliva should be a beneficial aid for oral health. In addition, we found that the acid would be able to increase salivary hBD2 concentration together with saliva secretion.

Based upon these facts, we firstly examined which acid is most potent to induce both salivary flow and hBD-2, using eleven kinds of acids which are allowed to be incorporated in the foods. The potential of acids to induce saliva were varied depending upon the kinds of acid (Fig. 2). Tartaric acid showed the highest potential, however, the potential of each acid to induce salivary hBD2 was quite different from that to induce saliva (Fig. 2). Citric acid was the most potent to induce hBD2 in saliva, followed by malic acid. Thus we employed citric acid in test gum to induce salivary hBD2. The concentration of salivary hBD2 was significantly increased.
4.2. Effects of Chewing Gum Containing Citric Acid (CA-gum) and Placebo Chewing Gum (Placebo Gum) on Oral Bacteria and Periodontal Pathogens

The chewing of either type of gum did not essentially affected the oral carriage of total cultivative bacteria (Wilcoxon t-test; p>0.05). Since more than 500 different kinds of resident bacteria form a normal flora in the oral region exposed to the action of the host defense system [14], it is assumed that commensal bacteria may possess certain mechanisms of escaping the host immune system, and pathogenic bacteria may not [15]. This may be one of the reasons why CA-gum, which induced salivary hBD2, did not affect the total bacterial counts.

It is reported that periodontal diseases are commonly associated with Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Dialister pneumosintes, Campy-
The effects of continual gum chewing of Placebo gum and CA-gum during 2 weeks on the oral carriage of P. intermedia. Thus in the present study, the oral carriage of P.g., P.i., T.f., T.d, and Fus. were examined as the representative periodontal pathogens.

P.g. is a gram-negative anaerobic bacterium involved in the pathogenesis of chronic adult periodontitis and is frequently found in the subgingival flora of diseased subjects [17]. This organism possesses a variety of virulence factors, including lipopolysaccharides, capsular material, fimbriae and proteases [18]. In the present study, placebo gum did not significantly reduce the P.g., whereas CA-gum tended to reduced P.g., but not significantly (Wilcoxon t-test; p>0.05). This was due to the fact that the frequency of the oral carriage of P.g. was very low; 4 of 31 subjects in placebo group and 8 of 30 subjects in CA group, respectively. Further study is necessary to clarify the effects of gum chewing on the oral carriage of P.g.

P.i. is a major periodontal pathogen [19] that is dominant in the periodontal pockets of patients with adult periodontitis [20,21]. This bacterium has also been frequently recovered from subgingival flora in patients with acute necrotizing ulcerative gingivitis [22] and pregnancy gingivitis [23]. Recently, it is reported that P. intermedia lipopolysaccharide

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Fig. (4). The effects of continual gum chewing of Placebo gum and CA-gum during 2 weeks on the oral carriage of P. intermedia.
promotes the production of NOx (nitrogen oxide) which might be important in the pathogenesis of inflammatory periodontal disease [24]. The frequency of the oral carriage of P.i. of each group was 17 of 31 subjects in placebo group and 15 of 30 subjects in CA group, respectively, and CA-gum significantly reduced the oral carriage of P.i. (Fig. 6; p<0.01), but this phenomenon was not observed with placebo gum (Fig. 4; Wilcoxon t-test; p>0.05).

T.f. is well known as a representative periodontal pathogen. It is reported that the metabolic products or a component of T.f. stimulates the growth of P.g. under nutrition-limited conditions and that the interaction between T.f. and P.g. in growth may be in part related with the synergistic virulence in a periodontitis model [25]. Further, a positive synergy between Fusobacterium and T.f. in the development of mixed biofilms has been reported [26]. These findings support the importance of T.f. in the development of periodontal disease. In the present study, T.f. was recovered from all participants in either the placebo or CA group. The oral carriage of T.f. was significantly reduced by CA gum (Fig. 5; p<0.01), but placebo gum did not (Wilcoxon t-test; p>0.05).

T.d. was found often in deep pockets, and the coinfection of P.g. Aa. and T.d. is reported to cause more serious periodontal destruction than infection of any one or two of the

**Fig. (5).** The effects of continual gum chewing of Placebo gum and CA-gum during 2 weeks on the oral carriage of *T. forsythensis*.
The effects of continual gum chewing of Placebo gum and CA-gum during 2 weeks on the oral carriage of *Fusobacterium* spp. Three microbes [27]. The frequency of the oral carriage of T.d. of each group was 21 of 31 subjects in placebo group and 22 of 30 subjects in CA group, respectively, and neither gum was effective to reduce the oral carriage of T.d. The phenomena were not surprising, since it has been recently reported that most strains or isolates of T.d. were resistant to hBD-1, 2 and 3 [28,29].

*Fuso.* is the most numerous gram-negative bacterium isolated from dental plaque biofilms [18,30]; it is a central species in biofilm development and a pathogen in human infections, including periodontitis. Important properties of *Fuso.* in biofilm development and pathogenesis include: the abilities to coaggregate to early and late colonizers of plaque biofilms [31,32]; adhere to and invade host tissue cells [33]; induce proinflammatory cytokines [34], and; produce proteases [35,36]. The frequency of the oral carriage of *Fuso.* of each group was 25 of 31 subjects in placebo group and 26 of 30 subjects in CA group, respectively, and CA-gum significantly reduced the oral carriage of *Fuso.* (Fig. 9; p<0.01). This phenomenon was not observed with placebo gum (Fig. 6; Wilcoxon t-test; p>0.05). The reduction of *Fuso.* by CA-gum should be beneficial not only for periodontal health but also for systemic health, since among the bacterial species associated with periodontal disease, *Fuso.* is the oral organism, most commonly found in systemic infections [30] and is strongly implicated in vaginal infections associated with pre-term deliveries [37].

Finally, our results, taken together, suggested that incorporation of citric acid into chewing gum reduced the periodontal pathogens in oral cavity, through increased salivary...
flow and increase in concentration of salivary hBD2, both being the beneficial aids for oral health. In addition, it is suggested that maternal or intrafamilial transmission has been considered a conceivable means of infection in children, and that the saliva is considered to be a major vector of transmission of periodontal pathogens [38]. Thus CA-gum chewing should be expected to be a helpful aid and tool to reduce the familial transmission of periodontal pathogens.

REFERENCES