The Intraday and Day-to-Day Fluctuation of the Antimicrobial Properties in Saliva as an Indicator of Resistance to the Oral Mucosal Disease

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Abstract
Possessing sufficient antimicrobial properties in saliva may prevent the excessive growth of fungus and intraoral indigenous bacteria to prevent the oral mucosal disease and pneumonia. The purpose of this study was to clarify the intraday and day-to-day fluctuations of three antimicrobial properties: beta-defensin, histatin, and IgA, which inhibit the growth of C. albicans and intraoral bacteria. Twenty healthy students or workers (10 males and 10 females; 25.7 ± 1.95 years) at Nihon University School of Dentistry at Matsudo were recruited. Resting saliva was collected daily from 10 to 11 AM, and from 15 to 16 PM, for seven days. By using ELISA, individual concentrations (ng/mL) of the antimicrobial properties were examined. The two-way ANOVA analyzed the effect of day-to-day and intraday fluctuations. No significant day-to-day fluctuations in beta-defensin (p = 0.13), histatin (p = 0.22), and IgA (p = 0.36) were detected. Also, no significant intraday fluctuations of beta-defensin (p = 0.58) and histatin (p = 0.70) were detected. However, IgA showed significant low concentration in the morning (p = 0.002) than in the afternoon. The results indicate that the saliva sampling in the morning, rather than the afternoon, is optimal for future studies of host immune factors: beta-defensin, histatin, and IgA.

Introduction
According to the annual report on the aging society 2018 from the cabinet office of Japan, pneumonia accounts for 95.3% of deaths in individuals over the age of 65 years. The primary cause of aspiration-related pneumonia is a pneumococcal infection, but it is also caused by fungus; e.g., Candida albicans, and intraoral indigenous bacteria; e.g., Staphylococcus aureus, which is formed on the dentures via biofilm. Thus, the biofilms on the teeth, oral mucosa and the dentures have to be removed by various mechanically and chemically methods, in general. (1-4) Although denture cleaning methods have been promoted in the dental community, the number of patients with aspiration-related pneumonia is still high. (5) Thus, in addition to general cleaning methods, preventive methods to reduce infections by C. albicans and intraoral indigenous bacteria are in need.

From this point of view, we focused on the host immune factors.

The antimicrobial properties of saliva in the oral cavity, which includes host immune factors reduce the infections of C. albicans and intraoral indigenous bacteria. (6-9) Antimicrobial factors in saliva include mucin, lactoferrin, lysozyme, peroxidase, histatin, cystatin, secretory white blood cell protease inhibitor, beta-defensin, and secretory IgA. Among these immune factors, we focused on histatin 5, beta-defensin 3, and IgA, which inhibit the growth of C. albicans and intraoral indigenous bacteria (6-9). The main reason that we focused on beta-defensin 3 was that it shows a specific effect on C. albicans and the majority of compositions in the saliva is beta-defensin 3. Regarding histatin, we followed the report by Vylkova et al. that histatin 5 was the one that showed a specific effect on C. albicans. Aiming to examine and identify the individual risk

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of infectious disease, this primary fundamental study purposed to test the reliability of day-to-day and intraday fluctuations of the immune factors; histatin, beta-defensin, and IgA. We think that it is preferable that there is no influence of time or date to obtain stable results in the examination of saliva sampling time. Although IgA levels are known to be higher in the afternoon than in the morning (10), intraday fluctuations in beta-defensin (with strong antimicrobial action) and histatin are unclear. The propose of this study was to seek the effect of intraday and day-to-day fluctuations to determine the optimal times to collect the saliva to beta-defensin, histatin, and IgA. This study will lead to future assessments of individual conditions in the oral immune system.

Materials and Methods

Participants

The participants were recruited from healthy students or workers at the Nihon University School of Dentistry at Matsudo. Twenty healthy students or workers, 10 men (mean ± standard deviation [range]: 26.4 ± 1.65 [25–30] years old) and 10 women (25.0 ± 2.05 [22–28] years old), were enrolled as participants. The participants were enrolled after providing written informed consent. Individuals were excluded if they (i) could not breathe through the nose; (ii) had oral lesions, such as candidiasis, recurrent herpes labialis, recurrent aphthous stomatitis, erythema migrans, hairy tongue, or lichen planus; (iii) had abnormal salivation of <0.1 mL/min; (iv) received medication; (v) had an autoimmune disease. This study was approved by the Human Ethics Committee of Nihon University School of Dentistry at Matsudo (#EC16-019).

Protocol for saliva collection

Resting saliva was collected twice a day in the morning from 10:00 to 11:00, and in the afternoon from 15:00 to 16:00 for 7 days by the spitting method. (11) Participants were asked not to eat or drink 2 hours before the saliva collection and not to brush their teeth or rinse their mouths 30 min before saliva collection.

ELISA

Saliva frozen until measurement. When we used ELISA method, saliva was dissolved and used saliva pretreatment with Tween 20 and HCl. Anti-beta-defensin 3 and histatin 5 levels in saliva were determined by using a beta-defensin 3 ELISA kit (BD-3, Human, ELISA Mini Development Kit; Peprotech, Rocky Hill, NJ, USA) and the Histatin 5 ELISA Kit (Cusabio, Baltimore, MD, USA) according to the manufacturers’ protocols. IgA levels in saliva were determined using the sandwich method. In this case methods, anti-IgA antibody was coated instead of Total Ig in order to improve the sensitivity of IgA in saliva. First, 96-well Falcon Microtest Assay Plates (BD Biosciences) were coated with 100 ng/mL Goat Anti Human IgA UNLB (Southern Biotechnology Associates, Birmingham, AL, USA) in PBS and incubated overnight at 4°C. Washing out unbound Goat Anti Human IgA UNLB 3 times with 200 μL of PBS containing 1 mL/L Tween 20. After blocking with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) in PBS, 55000-fold dilutions of Anti-IgA (α-chain) (Human) pAb-HRP (Medical & Biological Laboratories Co., Nagoya, Japan) and 100000-fold dilutions of samples were added and incubated for 24 hours at 4°C. After washing out unbound proteins, Goat Anti Human IgA (H + L) HRP (Southern Biotechnology Associates) was added to individual wells and incubated for 4 h at room temperature. The color reaction was developed for 15 min at room temperature with 100 μL of 1.1 mM ABTS (EMD Biosciences, La Jolla, CA, USA). Absorbance at 415 nm was measured using a microplate reader (CORONA ELECTRIC Co., Ibaraki, Japan).

Statistical analyses

Normal distribution of the data was tested with the Kolmogorov–Smirnov test, and parametric statistical analysis was applied. Two-way analysis of variance (ANOVA) was used to evaluate the effect of intraday and day-to-day fluctuation in beta-defensin 3, histatin 5, and IgA. All statistical analyses were performed using IBM SPSS Statistics 21 (IBM Corp., Armonk, NY, USA), and p<0.05 was considered statistically significant.

Results

Beta-defensin 3

Figure 1 shows beta-defensin 3 levels (mean ± SD ng/mL) from Monday through Sunday. No significant difference was observed in day-to-day beta-defensin 3 concentration (Monday: 41.6 ± 35.9, Tuesday: 38.2 ± 39.3, Wednesday: 36.6 ± 31.6, Thursday: 30.2 ± 27.8, Friday: 36.1 ± 36.2, Saturday: 44.22 ± 36.1, and Sunday: 44.1 ± 41.8, respectively). No significant difference was observed in intraday day beta-defensin 3 levels (in the morning: 38.0 ± 32.6 and in
Histatin 5

Figure 1 shows histatin 5 levels (mean ± SD ng/mL) from Monday through Sunday. No significant difference was observed in intraday day histatin 5 concentration (in the morning: 20.5 ± 23.0 and in the afternoon: 21.6± 22.8).

IgA

Figure 1 shows IgA levels (mean ± SD ng/mL) from Monday through Sunday. No significant difference was observed in day-to-day IgA concentration (Monday: 7.96 ± 5.43, Tuesday: 10.4 ± 11.3, Wednesday: 10.5 ± 10.6, Thursday: 6.37 ± 3.74, Friday: 8.46 ± 7.48, Saturday: 8.43 ± 8.73, and Sunday: 8.17 ± 8.32, respectively). IgA levels obtained in the morning and afternoon were 5.56 ± 3.58 and 11.6 ± 13.4 (10^4 ng/mL), respectively. There were significant intraday differences in the salivary IgA, lower concentration in the morning (5.56 ± 3.58) compared to those in the afternoon (11.6 ± 13.4) (p=0.002).

Discussion

This study was purposed to clarify the intraday and day-to-day fluctuations of three antimicrobial properties: beta-defensin, histatin, and IgA, which inhibit the growth of C. albicans and intraoral bacteria. The results indicate that intraday and day-to-day fluctuations had not influenced beta-defensin and histatin. IgA showed significant intraday differences: lower concentration in the morning compared to those in the afternoon.

The secretory immunoglobulin in saliva is sensitive to psychological variables and psychological stress, which humans are exposed to daily (12, 13). In general, the stress differs between the weekdays and weekends. Thus, secretion of immunoglobulin may differ between those periods. In this study, however, the results indicated that there was no influence of day-to-day effect, i.e., the weekdays and weekends. When testing the individual potentiality of resistance to oral infection, the result of beta-defensin or histatin can be reliable without the concern of saliva collection time. In contrast, the individual variation was high as seen from high standard deviation against the mean value. The individual within day-to-day difference is unknown from this result and should be clarified in the future research. Nevertheless, within this limitation, the measurement of immunoglobulin could be suggested as stable in average throughout the week.

With intraday fluctuations, Shinada et al. reported that IgA in saliva showed the highest value at immediately after the afternoon: 39.5 ± 38.4).

Histatin 5

Figure 1 shows histatin 5 levels (mean ± SD µg/mL) from Monday through Sunday. No significant difference was observed in day-to-day histatin 5 concentration (Monday: 25.6 ± 29.0, Tuesday: 22.8 ± 27.7, Wednesday: 15.9 ± 17.7, Thursday: 24.9 ± 23.6, Friday: 21.3 ± 23.4, Saturday: 18.9 ± 18.5, and Sunday: 17.9 ± 20.5, respectively). No significant difference was observed in intraday day histatin 5 concentration (in the morning: 20.5 ± 23.0 and in the afternoon: 21.6± 22.8).
waking and decreased rapidly and became stable after 10:00 a.m. The result of this study also showed stable IgA concentration after 10:00 a.m. and the values examined in the morning were kept constant in a week. However, IgA showed significantly higher values in the afternoon compared to the values in the morning. This agrees with the report of Guo et al. According to the value of IgA described in the textbook, the afternoon levels of this study were comparable. The subjects' background could influence the difference between Shinada et al.; their background was five female workers, and the studied subjects were 20 cross-gender postgraduate students and residents. This might lead to the different intraday fluctuations of IgA secretion.

From the results of intraday and day-to-day fluctuations, the knowing the ability of individual host immune factors to prevent various oral disease related to remaining biofilms, we assume that examining the low values of immune factors to specify the risk holders to infection. Thus, based on the results, the variance of intraday IgA is to be focused, and saliva sample in the morning, if possible from 1000 to 11:00, may be preferable to detect a state which the resistance of an individual's IgA is sensitively low.

There are several limitations to this study. Because the study subjects were comparatively young, the result of this study may not be adapted to the elderly, and study with the aged population should be investigated including age and living environment in the future study. Secondly, we need to measure saliva flow rate, but we have not measured this study. However, since there is no significant difference in the week, and the intraday fluctuation is consistent with other reports, we believe that the results of this study are reproducible. Third, the biofilms of the subject do not include those adhere to prostheses. The final goal of the study is focused on the elderly. Thus the effect of the biofilms attached to various organs and prostheses in the oral region should be in an account in the future study. Nevertheless, within this limitation, our results indicated that saliva sampling in the morning, rather than the afternoon, is optimal for future studies of host immune factors: beta-defensin, histatin, and IgA.

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Conflict of interest
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