Relationship among salivary antioxidant activity, cytokines, and periodontitis: the Nagasaki Island study


Abstract

Aim: Antioxidant activities and cytokine levels in human body fluids are considered to be strongly associated with periodontitis. The aim of this study was to elucidate the relationship between salivary antioxidant activities against superoxide or hydroxyl radical, cytokines, and periodontal conditions through a community-based cross-sectional study conducted in Goto city, Japan.

Materials and Methods: Saliva samples were analysed for superoxide or hydroxyl radical scavenging activities and cytokine levels from 160 participants. We demonstrated that saliva contained superoxide and hydroxyl radical scavenging activities by using electron spin resonance with a spin-trapping agent. The concentrations of eight cytokines were measured using multiplex bead assays.

Results: There were significant differences in salivary superoxide or hydroxyl radical scavenging activity and cytokine levels from 160 participants. We demonstrated that saliva contained superoxide and hydroxyl radical scavenging activities by using electron spin resonance with a spin-trapping agent. The concentrations of eight cytokines were measured using multiplex bead assays.

Conclusions: These findings suggest that the evaluation of salivary antioxidant activities, as assessed by electron spin resonance, are associated with periodontitis and various clinical variables in community-dwelling participants (ClinicalTrials.gov number NCT01742728).

Periodontitis is an inflammatory disease that starts in the biofilm. In this disease, the stimulation of the host defence system against bacterial pathogens plays an important role in the progression of tissue destruction. During periodontitis, reactive oxygen species (ROS) and cytokines that are released from immune cells to fight periodontal pathogens diffuse into the blood stream. The overproduction of ROS and parallel decrease in antioxidant power leads to oxidative stress, which is believed to be one of the causes of systemic disease (Halliwell 2012). It has also been suggested that systemic increases in ROS are involved in the pathogenesis of periodontitis (Chapple & Matthews 2007). For instance, a positive
association has been shown between systemic oxidative stress, pocket depth (D’aiuto et al. 2010), and the attachment level of chronic periodontitis patients (Tamaki et al. 2008). In addition, a linear correlation between the antioxidant activities of saliva against specific ROS and periodontal conditions was indicated; however, systemic factors and cytokines were not considered (Yoshino et al. 2012).

Cytokines and growth factors in human body fluids have been often associated with periodontitis. The analysis of inflammatory mediators in whole saliva provides a more global measure of inflammation throughout the mouth (Kaufman & Lamster 2000). Whole saliva is composed of constituents derived from the salivary glands, gingival crevicular fluid (GCF), epithelial cells, and bacteria. The cytokines and growth factors in whole saliva are mainly from the periodontium, via an influx of GCF. Therefore, the analysis of saliva provides a general assessment of oral inflammatory burden, which may in turn be a measure of the influence of periodontitis. Based on these recent results, we hypothesized that the classification of periodontitis or periodontal parameters would affect the activities of salivary antioxidants against specific ROS, such as superoxide or hydroxyl radical, and cytokine levels. Furthermore, salivary antioxidant activities may be candidates for new indexes for the diagnosis of periodontitis. The aim of this study was to elucidate the relationship between salivary antioxidant activities and periodontal conditions through a community-based study.

Materials and Methods

Study population

In 2010, 362 participants attended a mass health examination in Goto City, Nagasaki Prefecture, Japan. This included a periodontal assessment and the completion of a detailed medical questionnaire, which included the recording of smoking and alcohol consumption habits. Participants were excluded from the study if they were <40 years old or had <15 teeth. Examination of 160 randomly selected individuals was undertaken due to the availability of resources. All subjects provided a signed informed consent prior to participation in this study. The study was approved by the Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences (No. 090528160) and was performed in accordance with the Declaration of Helsinki. The study complies with the STROBE statement (see http://strobe-statement.org/).

Dental examination

One of four calibrated dentists (HH, MK, KK, TS) carried out each periodontal examination under sufficient illumination using artificial light. The probing pocket depth (PPD) and clinical attachment loss (CAL; the distance from the cementoenamel junction to the bottom of the pocket) were measured using a periodontal probe at the mesiobuccal and midbuccal sites for all the teeth present, excluding the third molars (Saito et al. 2008). Periodontal status was classified as healthy or mild, moderate, or severe periodontitis as proposed by the Center for Disease Control and Prevention in partnership with the American Academy of Periodontology (Eke et al. 2012). Severe periodontitis was defined as the presence of 2 or more interproximal sites with ≥6 mm AL (not on the same tooth) and 1 or more interproximal site(s) with ≥5 mm PD. Moderate periodontitis was defined as 2 or more interproximal sites with ≥4 mm clinical AL (not on the same tooth) or 2 or more interproximal sites with PD ≥5 mm, also not on the same tooth. Mild periodontitis was defined as ≥2 interproximal sites with ≥3 mm AL and ≥2 interproximal sites with PD ≥4 mm PD (not on the same tooth) or 1 site with ≥5 mm.

Saliva collection

Whole saliva samples were collected from 9:00 to 11:00 am. The participants were required to abstain from eating and drinking for 2 h prior to saliva collection. Salivette sampling devices (Sarstedt, Rommelsdorf, Germany) were placed under the tongue for 1 min to collect unstimulated saliva (Topkas et al. 2012). The samples were placed on ice before centrifugation. To extract the saliva, the swabs were centrifuged according to manufacturer’s protocol, and flow rates were determined. The divided supernatants were stored at −80°C until analysed.

Salivary antioxidant activity

Superoxide was generated by hydrogen peroxide (100 μM) and titanium dioxide photocatalysis, and hydroxyl radical was generated by ultraviolet irradiation of hydrogen peroxide (100 mM) as described previously (Yoshino et al. 2012). Electron spin resonance (ESR) spin trapping was conducted with a ROS-generating system containing 5-(2,2-dimethyl-1,3-propanediol)-5-methyl-1-pyrroline-N-oxide (Radical Research, Tokyo, Japan) (Kamibayashi et al. 2006). ESR observations were performed using a JES-REX1 ESR spectrometer (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR Data Analyzer (Radical Research) using the following instrument settings: microwave power, 8.00 mW; magnetic field, 335.6 ± 7.5 mT; field modulation width, 0.079 mT; sweep time, 1 min; and time constant, 0.03 s. In the control group, saline was added instead of saliva (Yoshino et al. 2012). For each experiment group, superoxide or hydroxyl radical scavenging activities (inhibition percentage) were calculated as 100% with a mean value of control respectively.

Multiplex cytokine analysis

Saliva samples were filtered through 0.22-μm filter spin filters (Millipore, Darmstadt, Germany) prior to cytokine analysis. Multiplex bead-based kits (Bio-Rad, Hercules, CA, USA) were used to simultaneously measure the concentrations of eight cytokines. The lower limits of each cytokine are as follows: interleukin (IL)-1β (1.9 pg/ml), IL-6 (1.5 pg/ml), IL-8 (1.6 pg/ml), IL-10 (0.6 pg/ml), interferon gamma (IFN-γ) (2.1 pg/ml), tumour necrosis factor-alpha (TNF-α) (1.5 pg/ml), platelet-derived growth factor (PDGF) (1.2 pg/ml), and vascular endothelial growth factor (VEGF) (2.3 pg/ml). The concentrations of these cytokines were...
measured in 96-well plates, using 50 μl per well of saliva, according to the manufacturer’s instructions. Plates were washed using the Bio-Plex Pro wash station (Bio-Rad) for magnetic beads, and standards and samples were assayed using the Bio-Plex system 200 (Bio-Rad) as previously reported (Khan 2012).

**Results**

**Study population**

A total 160 individuals participated in this study. The majority of participants consisted on female (ratio female/male: 110/50), with age ranging between 40 and 84. The participants with smoking (current or past) were 34 (21%) and with alcohol consumption habit were 32 (20%). In addition, half of participants had hypertension (45%), and less often diagnosed as having diabetes mellitus (4%).

**Salivary antioxidant activities and cytokines**

We obtained the typical ESR spectra of control for superoxide or hydroxyl radical scavenging activities (Fig. 1A, C). When saliva was applied, the reduction of peak was detected (Fig. 1B,D). In addition, the median (inter-quartile range 25%, 75%) of salivary superoxide or hydroxyl radical scavenging activities by ESR were 44 (32, 56) and 66 (56, 80), respectively. The results from the multiplex bead-based analysis showed that most cytokines in saliva were within the ranges of quantification. For IL-6, IL-10, and TNF-α, over 80% of the samples were within the range. In the case of IFN-γ and PDGF, more than 60% of the samples were quantifiable.

**Comparison of salivary parameters between the classifications of periodontitis**

Participants were classified into three groups: severe periodontitis (n = 25), moderate periodontitis (n = 43), and healthy to mild periodontitis (n = 92). There were significant differences in superoxide or hydroxyl radical scavenging activity (p < 0.001), IL-1β (p < 0.001), IL-6 (p = 0.002), and IL-8 (p = 0.003) between the groups (Table 1). No significant differences were detected between classifications of periodontitis for gender, alcohol consumption habit, hypertensions, and diabetes mellitus. The percentage of smokers tended to be higher in the severe periodontitis group, although it did not reach to the statistically significant level (p = 0.057).

In addition, a multiple logistic regression model is presented in Table 2. We divided the participants into two groups, depending on high or low salivary antioxidant activities. As the cut-off points, each median...
level of antioxidant activity (%) by ESR spectroscopy was used; 44% for superoxide scavenging activity and 66% for hydroxyl radical scavenging activity. The final model showed the classification of periodontitis (moderate to severe periodontitis versus others) was significantly associated with salivary superoxide scavenging activity (odds ratio: 2.612; 95% confidence interval: 1.286–5.304, *p* = 0.008) and hydroxyl radical scavenging activity (odds ratio: 2.695; 95% confidence interval: 1.330–5.461, *p* = 0.006). Salivary IL-1β, IL-6, IL-8, VEGF, age, and smoking status were excluded from explanatory variables.

### Associations between periodontal conditions and each parameter

For evaluation of the relationships between periodontal parameters (mean PPD, mean CAL, and BOP) and salivary antioxidant activities or salivary cytokines, variables were selected for the independent variables of the final models. The results of multiple linear regression analyses by the final model are presented in Table 3.

**Table 1.** Clinical parameters in different periodontal status

<table>
<thead>
<tr>
<th></th>
<th>Healthy to Mild periodontitis (n = 92)</th>
<th>Moderate periodontitis (n = 43)</th>
<th>Severe periodontitis (n = 25)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean (SD)</strong>*</td>
<td><strong>Age (years)</strong></td>
<td>62.1 (10.6)</td>
<td>66.1 (7.3)</td>
<td>66.6 (10.3)</td>
</tr>
<tr>
<td></td>
<td><strong>Teeth present (n)</strong></td>
<td>24.4 (3.6)</td>
<td>23.4 (3.8)</td>
<td>23.4 (4.0)</td>
</tr>
<tr>
<td></td>
<td><strong>Mean PPD (mm)</strong></td>
<td>1.3 (0.3)</td>
<td>1.6 (0.4)</td>
<td>2.0 (0.5)</td>
</tr>
<tr>
<td></td>
<td><strong>Mean CAL (mm)</strong></td>
<td>2.0 (0.5)</td>
<td>2.6 (0.6)</td>
<td>3.5 (1.2)</td>
</tr>
<tr>
<td></td>
<td><strong>BOP (%)</strong></td>
<td>7.9 (9.0)</td>
<td>11.2 (11.4)</td>
<td>19.1 (16.7)</td>
</tr>
<tr>
<td></td>
<td><strong>Superoxide scavenging activity (%)</strong></td>
<td>39.1 (21.8)</td>
<td>48.3 (16.6)</td>
<td>57.8 (18.3)</td>
</tr>
<tr>
<td></td>
<td><strong>Hydroxyl radical scavenging activity (%)</strong></td>
<td>61.5 (15.6)</td>
<td>70.4 (15.3)</td>
<td>77.7 (15.1)</td>
</tr>
</tbody>
</table>

**Table 2.** Relationship between the classification of periodontitis and salivary biomarkers by stepwise logistic regression analysis

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Adjusted OR</th>
<th>95% CI</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide scavenging activity</td>
<td>&lt;44</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥44</td>
<td>2.612</td>
<td>1.286–5.304</td>
</tr>
<tr>
<td>Hydroxyl radical scavenging activity</td>
<td>&lt;66</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥66</td>
<td>2.695</td>
<td>1.330–5.461</td>
</tr>
</tbody>
</table>

OR, odds ratio adjusted for all variables; CI, confidence interval.

### Relationship between salivary antioxidant activities and periodontal parameters

The association between salivary antioxidant activities and periodontal parameters are shown in Fig. 2. The mean PPD (*p* < 0.001), mean CAL (*p* < 0.001), and BOP (*p* < 0.001) for participants in the high superoxide scavenging activity group were significantly higher than those in the low group. Similarly, participants with high hydroxyl radical scavenging activity group had a higher mean PPD (*p* < 0.001) and mean CAL (*p* < 0.01). No significant difference in BOP was observed in participants in the hydroxyl radical scavenging activity group.
scavenging activity ($\beta = 0.262; p = 0.003$) adjusted for diabetes mellitus and gender. In addition, mean CAL was positively associated with saliva levels of IL-10 ($\beta = 0.152; p = 0.041$), and was even more significantly associated with superoxide scavenging activity ($\beta = 0.346; p < 0.001$). There was a negative association between salivary VEGF concentration and mean CAL ($\beta = -0.209; p = 0.022$). According to the results of BOP, only superoxide scavenging activity had a significant effect ($\beta = 0.408; p < 0.001$).

**Discussion**

The results of our study demonstrate significant differences in the salivary antioxidant activities and some cytokine concentrations for different classifications of periodontitis. In addition, multiple logistic regression model revealed that salivary superoxide and hydroxyl radical scavenging activities were significantly correlated with the classification of periodontitis. The high salivary superoxide scavenging activity group showed an increased index of periodontal conditions. Similarly, participants in the high hydroxyl radical scavenging activity group had higher mean probing pocket depth and clinical attachment loss. Furthermore, salivary superoxide scavenging activity was found to have a significant association with all periodontal parameters (mean PPD, mean CAL, and BOP) using multiple linear regression analysis. Likewise, salivary hydroxyl radical scavenging activity and a number of cytokines were also related to particular periodontal parameters. Overall, salivary antioxidant activity was the parameter more strongly related to periodontitis than salivary cytokines.

ROS play important roles in physiological and immunoinflammatory reactions (Waddington et al. 2000). Leucocytes kill bacteria by producing ROS such as superoxide. However, excessive ROS induces oxidative damage in host cells. Despite its cytotoxic effect, superoxide plays important physiological roles. Superoxide molecules function as regulatory signal molecules that are involved in numerous signal transduction cascades (Sauer et al. 2001) and also regulate biological processes (Liu et al. 2005). In addition, excessive levels of superoxide reduce transition metal ions; the reduced forms of these ions in turn react with hydrogen peroxide to produce hydroxyl radicals. From the oxidant species, the hydroxyl radical has the strongest effect and reacts indiscriminately with nucleic acids, proteins, and lipids. However, a detoxification system for hydroxyl radicals is rarely observed. Subjects with worse periodontal condition tended to have increased oxidative injury to periodontal tissue. Recently, total levels of ROS or antioxidant activity were determined using fluorescence analysis.
The use of saliva samples provides many advantages over blood. The collection of whole saliva does not require any invasive procedures, which can potentially affect experiments. Whole saliva is composed of fluid derived from salivary glands and gingival crevicular fluid, and the antioxidant activities in whole saliva are still unclear. Further study is required to clarify the relations between salivary antioxidant activities and periodontitis.

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However, such data are scarce. Moreover, a previous study indicated that the levels of IL-1β, IL-8, IL-10, and TNF-α were higher and the levels of other cytokines were almost equal in saliva compared to plasma or urine (Khan 2012). Therefore, saliva samples could represent a non-invasively obtained, sensitive, and useful fluid for the assessment of periodontal disease. For these reasons, we chose to use saliva in our analysis of the relationship between periodontal disease, antioxidative activities, and the concentrations of various cytokines.

Imunoassays have been the most popular techniques for detecting and quantifying cytokines or growth factors in body fluids. The major drawbacks of enzyme-linked immunosorbent assays or immunoassays are the relatively low throughput and the requirement for a large amount of sample for many kinds of analyses. In this regard, the new technology of multiplex analysis has emerged as a useful tool for candidate biomarker screening (Khan 2012). The major advantages of multiplex analysis are decreased experimental variability, simultaneous detection of numerous cytokines from low sample volume, the generation of quantitative results, and cost effectiveness (Lee et al. 2006). Using this form of analysis on saliva samples, the concentrations of eight cytokines were obtained from only one 96-well assay plate. Therefore, we have measured levels of salivary cytokines by using multiplex analysis.

Periodontal diseases induce local and systemic elevations of pro-inflammatory cytokines, resulting in tissue destruction. Previous study showed that salivary cytokines offer potential for the prediction of periodontal disease progression (Kinney et al. 2011). IL-1β and IL-6 are pro-inflammatory cytokines produced by macrophages, monocytes, fibroblasts, and dendritic cells during the tissue inflammatory response to various stimuli, including microbial challenge (Baumann & Gauldie 1994). In this study, salivary VEGF levels were negatively associated with attachment loss. VEGF is multifunctional angiogenic cytokines that are important in inflammation and wound healing (Booth et al. 1998).

It has been also suggested that periodontal treatment decreases BOP and increases the level of IFN-γ, which is involved in immune responses and induces mainly cell-mediated responses (Del Pelosi Ribeiro et al. 2008). Similarly, salivary IL-8 is a key chemokine mediating neutrophil migration (Teles et al. 2009). Porphyromonas gingivalis, one of the major periodontopathic bacteria, induces the release of cytokines such as TNF-α, IL-6, and IL-10 (Berkner et al. 2005). These cytokines increase the number of polymorphonuclear leucocytes and their activation (Calkins et al. 1998). It is well known that ROS are produced by activated polymorphonuclear leucocytes and then flooded into the bloodstream (Mittal et al. 2014). In addition, ROS also lead to the increase in antioxidative activity. In our present study, the positive associations between salivary antioxidative activities and some of cytokine were indicated.

In this study, all clinical parameters, salivary antioxidative activities, and cytokines were coherent because statistical associations were revealed by multiple linear regression analysis. These data may be interpreted as identifying favourable parameters for the estimation of periodontal disease. However, the results of this study should be described with caution as a community-based study with a cross-sectional design was used rather than a longitudinal study design. Therefore, it is still uncertain whether periodontal disease is the cause or the result of increased salivary antioxidative activities against superoxide and hydroxyl radical. Further comprehensive studies are required to determine the effects of periodontal treatment using salivary samples. It will also be interesting to see if these results can be confirmed in severe periodontitis patients, as this would add to our understanding of how salivary antioxidative power and cytokines contribute to the progression of periodontitis. Furthermore, we predict that longitudinal monitoring of salivary antioxidative activities will verify the results of our study.

In conclusion, our data suggest that salivary antioxidative activities against specific ROS, as assessed by ESR, were associated with the classification of periodontitis and various clinical periodontal parameters in community-dwelling participants. In particular, the quantification of salivary superoxide scavenging activity was associated with periodontal parameters more strongly than any of the biomarkers, and may be a better biomarker for predicting periodontitis than salivary cytokines.

Acknowledgements

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References


Tamaki, N., Takaki, A., Tomofuji, T., Endo, Y., Kasayama, K., Ekuni, D., Yasunaka, T., Yamamoto, K. & Morita, M. (2011) Stage of hepatocellular carcinoma is associated with reactive oxygen species can be useful biomarker for periodontitis. In particular, the quantification of salivary superoxide scavenging activity would be a better biomarker for predicting periodontitis than salivary cytokines.