Age-related changes in salivary biomarkers

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Received 26 September 2013; Final revision received 26 October 2013
Available online 6 February 2014

Abstract  Background/purpose: Saliva plays a critical role in the oral cavity health; the levels of its constituents alter with age. The reduced glutathione:oxidized glutathione ratio (GSH:GSSG) in the plasma is reported to be lower in elderly people and thus can be an indicator of age. The aim of this study was to detect age-related changes in salivary biomarker levels and evaluate whether the salivary GSH:GSSG ratio can be an indicator of aging.

Materials and methods: Individuals who participated in this study were divided into two groups: the elderly group (n = 20; age 60–80 years) and the young group (n = 20; age 20–30 years). Unstimulated saliva was collected passively for 5 minutes, followed by clinical examination. The salivary flow rate (SFR), pH, and buffering capacity were measured, followed by centrifugation of saliva, collection of supernatant, and measurement of the following biomarkers: calcium (Ca), alpha (α)-amylase, GSH, GSSG, matrix metalloproteinase-8 (MMP-8), collagenase type-I, and tissue inhibitor of metalloproteinase (TIMP-1). Descriptive analyses of variables were performed.

Results: The elderly group showed significantly lower SFR and Ca than the young group, whereas collagenase type-1 and MMP-8 were significantly lower in the young group. None of pH, buffering capacity, α-amylase, GSH, GSSG, GSH:GSSG, or TIMP-1 showed any statistically significant difference between the two groups.

Conclusion: Saliva is a mixture of components, the levels of which can increase, decrease, or remain stable with age. Although the GSH:GSSG ratio was lower in the elderly group, it did not reach a level of significance.

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Introduction

The number of people aged over 60 years is growing faster than that of any other age group. Approximately 10% of the world’s population is over the age of 60 years; this number is likely to increase to 15% by the year 2050.1 Physiological changes occur with aging in almost all organ systems.2 It is widely accepted that saliva composition alters with age,3 which can manifest as low resistance of the elderly to oral diseases.4 Saliva is an essential fluid that maintains oral health and homeostasis by providing the necessary host defense functions.5 This fluid can be collected non-invasively and used as a diagnostic tool to provide information about the health or disease status of an individual.6 Despite this knowledge, the literature is controversial regarding the age-related changes in the salivary biomarker.

Among the many proposed theories of aging, free radical and oxidative stress theories received special attention.7 Glutathione is the most prevalent and most important intracellular thiol–disulfide redox buffer in mammalian cells; it exits in two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). GSH, the active form, is a water soluble tripeptide containing cysteine, glutamic acid, and glycine8,9; it accounts for around 90% of the intracellular thiols. The remaining 10% is made up of other small thiol compounds.10 The thiol group in cysteine is a potent reducing agent, rendering GSH an essential antioxidant in the detoxification of a variety of electrophilic compounds and peroxides.11 It is found in micromolar (μM) concentrations in body fluids and in millimolar (mM) concentrations in tissue. The GSH:GSSG ratio is indicative of oxidative stress and cellular health.12 It was reported that the GSH:GSSG ratio in the plasma is an indicator of aging.13 Calcium (Ca) is the fifth most abundant mineral in the human body and one of the most intensively studied minerals in saliva due to its role in dental and gingival health.14,15 Serum Ca ions were reported to be lower in healthy elderly persons than in healthy young adults.16 Alpha (α)-amylase is one of the most abundant components in human saliva that can play a role in oral health or disease. In solutions this enzyme binds to bacteria, contributing to the bacterial clearance. By contrast, in enamel pellicle, it initiates the digestion of starch, thus providing substrates for colonizing bacteria and enhancing their adherence to tooth surfaces.17,18 Both functions need an intact enzyme conformation.17 The changes in salivary Ca and α-amylase levels with age are still unclear.

To our knowledge, no existing studies have addressed the effect of aging on the GSH:GSSG ratio in saliva. Thus, the aim of this study was to evaluate the changes in the levels of certain markers of unstimulated saliva with age. The tested null hypothesis was that no difference was observed in the levels of salivary biomarkers between the elderly and young groups.

Materials and methods

This study was carried out in 20 healthy young adults in the age group of 20–30 years and 20 healthy elderly people in the age group of 60–80 years, recruited from the Tokyo Medical and Dental University. Informed consent was obtained from all the study participants, and the study protocol was approved by the ethical committee of the Tokyo Medical and Dental University (number 701).

Prior to saliva collection, patients were allowed to rest for a few minutes and asked to rinse their mouth with water. They were seated comfortably, and asked to lean slightly forward and not to swallow. After 5 minutes, whole saliva was collected in a collection tube by passive drooling. Clinical examination was performed to record the number of decayed, missing, and filled teeth (DMFT).

The amount of saliva was measured and the salivary flow rate (SFR) was calculated (mL/5 minute). The pH and buffering capacity of saliva were measured immediately using a hand-held pH meter (Checkbuf; Horiba Ltd., Kyoto, Japan), followed by centrifugation of saliva for 10 minutes at 10,000 rpm (9,170 × g). The supernatant was collected and stored at −80 °C until further analysis of the following salivary biomarkers: Ca (AA-630, an atomic absorption spectrometer; Shimadzu Corporation, Kyoto, Japan), α-amylase (EnzyChrom, an α-amylase assay kit; BioAssay Systems, Hayward, CA, USA), GSH and GSSG (GSSG/GSH Quantification Kit; Dojindo Laboratories, Kumamoto, Japan), matrix metalloproteinase-8 (MMP-8; Quantikine Human Total MMP-8 ELISA Kit; R&D Systems Inc., Minneapolis, MN, USA), collagenase type-I (Type-I Collagenase Assay Kit; Primary Cell Co., Ltd., Sapporo, Japan), and tissue inhibitor of metalloproteinase (TIMP-1; Quantikine Human TIMP-1 Immunoassay; R&D Systems Inc.). Descriptive analyses of variables were performed. Variables of two groups were compared using Student t test, and the correlation was assessed using Pearson coefficient.

Results

Forty healthy individuals were divided into two groups according to their age. Table 1 represents the mean age and DMFT of each group. Table 2 shows the mean and the standard deviation of each evaluated salivary variable in the individuals tested. The SFR was statistically lower in elderly people (P < 0.001), whereas saliva pH (P = 0.998) and buffering capacity (P = 0.594) showed no statistically significant difference between the two groups. Neither the GSH (P = 0.599) nor the GSSG (P = 0.571) level showed a statistically significant difference between elderly and young adults. Although the GSH:GSSG ratio was higher in young individuals, it was not statistically significant.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Distribution of participants and DMFT in different age groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group</td>
<td>N</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
</tr>
<tr>
<td>Young group</td>
<td>20</td>
</tr>
<tr>
<td>Elderly group</td>
<td>20</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.

DMFT = decayed, missing, and filled teeth; SD = standard deviation.

a,b Different letters represent a significant difference (P < 0.05).
compared to that obtained in elderly people ($P = 0.14$). Salivary Ca level was significantly lower in the elderly when compared to young adults ($P < 0.015$) and collagenase type-I ($P < 0.001$) showed statistically higher values in elderly adults compared to young individuals.

Collagenase type-I showed a negative correlation with SFR ($P = 0.002$, $R^2 = 0.239$) (Fig. 1A). Both Ca ($P < 0.001$, $R^2 = 0.313$) and TIMP-1 ($P < 0.001$, $R^2 = 0.369$) showed a positive correlation with $\alpha$-amylase (Fig. 1B and C, respectively). A positive correlation was also found between MMP-8 and collagenase type-I ($P = 0.008$, $R^2 = 0.174$; Fig. 1D).

### Discussion

The current percentage of elderly people in Japan is 24.1%, and this number may escalate to 38% by 2050.\textsuperscript{19,20} This increase implies that dental practitioners will provide health care for more elderly people in the future; hence, they should be aware of the changes occurring in salivary composition with age. In this study, secretion of saliva and the levels of some of its components were found to be significantly different between the two age groups; this requires partial rejection of the null hypothesis.

Our results showed that the unstimulated SFR decreases with age, which is in accordance with previously published studies.\textsuperscript{21–23} This lower SFR was attributed to the physiologic process of aging and parenchymal atrophy in the salivary glands,\textsuperscript{24,25} and was suggested to be a causative factor for the increase in oral mucosal diseases seen in the elderly.\textsuperscript{22} Submandibular glands are responsible for 70% of the resting saliva flow.\textsuperscript{26} The cellular activity and secretory functions of this gland decrease with age.\textsuperscript{27,28}

### Table 2

Means and SDs of the evaluated salivary variables in the tested individuals.

<table>
<thead>
<tr>
<th>Salivary maker</th>
<th>Young individual</th>
<th>Elderly person</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (mL/5 min)</td>
<td>3.8 ± 1.3\textsuperscript{a}</td>
<td>2.1 ± 0.8\textsuperscript{b}</td>
</tr>
<tr>
<td>Buffering capacity</td>
<td>5.0 ± 1.1\textsuperscript{a}</td>
<td>5.2 ± 0.82\textsuperscript{a}</td>
</tr>
<tr>
<td>Calcium (mg/L)</td>
<td>41.1 ± 18.6\textsuperscript{a}</td>
<td>29.9 ± 17.4\textsuperscript{b}</td>
</tr>
<tr>
<td>$\alpha$-amylase (U/L)</td>
<td>6688 ± 2587\textsuperscript{a}</td>
<td>6363 ± 2478\textsuperscript{a}</td>
</tr>
<tr>
<td>GSH (µmol/L)</td>
<td>15.9 ± 23.9\textsuperscript{a}</td>
<td>12.3 ± 19.7\textsuperscript{b}</td>
</tr>
<tr>
<td>GSSG (µmol/L)</td>
<td>5.3 ± 9.2\textsuperscript{a}</td>
<td>3.8 ± 7.2\textsuperscript{b}</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>5.7 ± 5.6\textsuperscript{a}</td>
<td>4.2 ± 2.4\textsuperscript{a}</td>
</tr>
<tr>
<td>Collagenase type-1</td>
<td>0.21 ± 0.08\textsuperscript{a}</td>
<td>0.46 ± 0.17\textsuperscript{a}</td>
</tr>
<tr>
<td>MMP-8 (ng/mL)</td>
<td>1.15 ± 1.2\textsuperscript{a}</td>
<td>3.4 ± 3.8\textsuperscript{b}</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>88.1 ± 32.0\textsuperscript{a}</td>
<td>79.7 ± 34.7\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b} Different letters represent statistically significant difference ($P < 0.05$).

GSH = reduced glutathione; GSSG = oxidized glutathione; MMP-8 = matrix metalloproteinase-8; SD = standard deviation; TIMP-1 = tissue inhibitor of metalloproteinase.

Figure 1 Scattergrams representing the correlation between some of the tested salivary features and/or biomarkers: correlations between (A) collagenase type-I and salivary flow rate, (B) $\alpha$-amylase and calcium, (C) $\alpha$-amylase and TIMP-I, and (D) collagenase type-I and MMP-8. MMP-8 = matrix metalloproteinase-8; TIMP-1 = tissue inhibitor of metalloproteinase.
Buffering capacity of saliva, one of its most important parameters, neutralizes acid in the mouth; it depends on bicarbonate concentration.29,30 The buffering capacity as evaluated in our study is consistent with that in previously published studies, which showed that saliva buffering capacity was not related to age.31,32 Human parotid glands secrete much of the bicarbonate that enters the mouth.33 The function of the parotid gland was shown to be independent of age and its constituents remain unchanged during aging.34-36

Despite Ca being one of the most studied minerals, there is no consensus on the effect of age on salivary Ca levels. One study showed that salivary Ca levels increase with age,37 another did not report a significant difference,38 and Chauncey et al39 found a diminished level of Ca in elderly males. Our result demonstrated that salivary Ca level declines with age; this is inconsistent with the result obtained by Sevon et al,37 who detected elevated levels of salivary Ca in elderly patients, with a peak at around the age of 50 years. They discussed that an increased salivary Ca level may reflect a decreased bone density in their patients. A possible explanation for the difference between the results of our study and those of Sevon et al37 is that the ages of the tested individuals differed between these two studies. In their study, females in the age range of 30-59 years were examined, whereas in our study, the elderly group consisted of both sexes in the age range of 60-80 years. In late older ages, restricted dietary intake,40 less intestinal absorption of Ca,41 and/or minimal exposure to sunlight42 may contribute to reduced Ca levels in body fluids.41

Alpha-amylase is another saliva component the level of which shows no changes with age. Ben-Aryeh et al43 found lower levels of α-amylase in the elderly, whereas others found no significant difference39 or even increased levels of this enzyme.44 Salivary α-amylase is produced mainly by the parotid glands and is considered a marker of parotid saliva.45 It was reported that this gland can maintain its secretory function over the human life span;46 this may explain the result obtained in our study, in which no significant difference was found in the α-amylase level between young and elderly persons. Differences in the results between studies may be attributed to different methodologies, different age groups, and/or collection of stimulated or resting saliva.42 Another factor that can influence the results is stress that is inherently present in dental practice, and can be induced in patients by routine dental procedures or even regular checkup.47 Stress is known to increase salivary α-amylase levels.18

Interestingly, in our study, we found a positive correlation between Ca and α-amylase levels. The role of plant cell Ca in the activation and stabilization of α-amylase structure has been studied extensively.48 The proposed stabilization mechanism involves an interaction between cations and some negatively charged amino acid residues. This interaction maintains the three-dimensional structure of protein that is necessary for the functioning of this enzyme.17,49 Removal of Ca from the genus Bacillus leads to decreased thermal stability and enzymatic activity of α-amylase,50 or even to increased susceptibility of this enzyme to proteolytic degradation.51 Human salivary amylase has metal-binding characteristics. It has two metal ion binding sites, and only one site is selective to Ca. It has been shown that neither copper nor zinc can replace Ca in salivary α-amylase, and this is reflective of the stability of Ca-amylose binding, making this interaction unique.52

Despite a higher GSH:GSSG ratio in young individuals, it did not reach the level of significance when compared to elderly people. Young adults who participated in this study live in a modern city where levels of pollution and stress are high; these could enhance the oxidative stress in the body,53 leading to a lower GSH:GSSG ratio.12 The small sample size may also have an influence on the results. A recent study showed that the GSH:GSSG ratio in saliva is lower in patients with periodontitis. However, it was restored to normal levels upon therapy.54 Despite the high concentration of glutathione in gingival crevicular fluid, the source of glutathione in its two forms in saliva is yet to be investigated.55 Salivary glutathione levels may be an index of increased levels of oxidative stress and an indication of increased risk of oral diseases.56

The levels of both collagenase type-I and MMP-8 were significantly higher in elderly persons when compared to those in young individuals. TIMP-1 showed no significant difference between the two groups. Most of the collagenases in saliva are said to originate from degranulating neutrophils, entering the mouth through the gingival sulcus.57 The negative correlation between saliva and collagenase type-I in our study supports the nonsalivary origin of this enzyme, and the positive correlation between MMP-8 and collagenase type-I may reflect the same origin for these two enzymes. The origin of salivary TIMP-1 remains unknown. However, evidence suggests a nonplasma origin, with the major contribution from the parotid gland58; this may be supported by the positive correlation between TIMP-1 and α-amylase, as the latter is also secreted by the parotid gland.

Despite the limited number of individuals investigated in this study, changes have been detected in some of the components of saliva with age, whereas other components remained unchanged. Further studies are warranted to evaluate the importance of the salivary GSH:GSSG ratio in oral health. The presence of Ca in enamel pellicle is essential for remineralization, whereas α-amylase in the pellicle provides the necessary substrates for colonizing bacteria and enhancing their adhesion to tooth structure, thereby inducing demineralization; thus, studying the interaction between Ca and α-amylase in enamel pellicle can be promising for better understanding of the re/demineralization process.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This work was supported by a grant from the Japanese Ministry of Education and Global Center of Excellence “GCOE” Program, International Research Center for Molecular Science in Tooth and Bone Diseases.
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