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ORIGINAL ARTICLE

Comparison of the composition of oral mucosal residual saliva with whole saliva

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OBJECTIVE: Compared with whole saliva, residual saliva comprising the oral mucosal film shows a high protein concentration. The purpose of this study was to compare the composition of residual saliva with unstimulated and stimulated whole saliva in normosalivators.
MATERIALS AND METHODS: The composition of oral mucosal residual saliva in 30 healthy individuals was investigated and compared with that of whole saliva. The concentrations of total protein, secretory immunoglobin A (sIgA), lactoferrin, total carbohydrate, and sialic acid were examined. The activities of peroxidase, lysozyme and α-amylase were determined.
RESULTS: Residual saliva had higher levels of total protein and carbohydrate than whole saliva, with a higher carbohydrate to protein ratio in the residual saliva suggesting that salivary glycoproteins are concentrated on the oral mucosal surface. sIgA, lactoferrin and sialic acid were present as highly concentrated forms in residual saliva. The enzymatic activity of peroxidase in residual saliva was higher than that of whole saliva.
CONCLUSIONS: These concentrated carbohydrate and antimicrobials on the oral mucosal surface work for mucosal defence and could be used for targeting sites for the delivery of therapeutic agents.
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Keywords: residual saliva; composition; protein; carbohydrate

Introduction

Residual saliva (RS), empirically defined as the salivary film coating on oral tissue surfaces, protect the oral mucosa from feeling dry. The thickness of this salivary film correlates with wetness and varies with the intraoral tissue locations. The sensation of a dry mouth is perceived when there is an insufficient mucosal wetting (DiSabato-Mordarski and Kleinberg, 1996; Wolff and Kleinberg, 1998; Dawes, 2004), and a correlation between mucosal film thickness and resting whole salivary flow rate or the severity of dry mouth has been reported (Wolff and Kleinberg, 1998; Kleinberg *et al*, 2002; Lee *et al*, 2002).

Proteins and glycoproteins facilitate the main functions of RS as a moisture retainer, protective barrier, lubricant and a determinant in microbial colonization. The protein concentration of RS is significantly higher than that of unstimulated whole saliva (UWS) in both normosalivators and hyposalivators, with mucosal wetness correlated with protein concentration (Tabak *et al*, 1982; Levine *et al*, 1987; Won *et al*, 2001; Lee *et al*, 2002). Increased protein concentration has been proven to be the result of decreased volume of RS (Won *et al*, 2001; Lee *et al*, 2002).

Salivary glycoproteins play a major role in mucosal defence and provide the mucosal surfaces with viscoelastic properties (Tabak *et al*, 1982; Levine *et al*, 1987; Cohen and Levine, 1989; Schenkels *et al*, 1996). Notably, salivary mucins bearing high levels of carbohydrate may form complexes with other important salivary proteins that have antimicrobial activities (Biesbrock *et al*, 1991; Iontcheva *et al*, 1997; Soares *et al*, 2003). In this way, they might function as a vehicle to concentrate such and other molecules on the oral mucosal surface. This could lead to the composition of concentrated proteins present on the surface of the oral mucosa differing from that of whole saliva. To help understand the role of saliva in oral mucosal protection, the knowledge of protein and carbohydrate compositions of RS is necessary.

Our purpose in the present study was to compare the composition of RS with UWS and stimulated whole saliva (SWS) in normosalivators. Total protein and carbohydrate, sialic acid and several antimicrobial molecules were examined.

Materials and methods

Participants
The composition of UWS, SWS and RS was analysed in 30 healthy subjects (15 men and 15 women, with an age

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range of 20–26 years and a mean age of 23.3 years). All subjects had no history of serious illness and conditions such as Sjögren's syndrome and irradiation, did not take any medication known to affect salivary flow rate for at least 3 months, and had no complaints suggestive of salivary gland dysfunction (Fox *et al*, 1987). The research protocol was approved by the Institutional Review Board of the University Hospital (no. CRI05019).

Collection of whole saliva and residual saliva
Saliva samples were collected twice from each subject to provide sufficient RS for analysis. The first sample was used for analysis of total protein, sIgA and α-amylase, while total protein, total carbohydrate, sialic acid, lactoferrin and peroxidase, and lysozyme activities were analysed with the second sample. All values were normalized to the total protein concentration of the sample analysed (total carbohydrate concentration in sialic acid).

Unstimulated whole saliva samples were harvested first, followed by RS and SWS. All samples were collected between 08:00 and 11:00 hours to minimize any effects of diurnal variability in salivary composition. Samples were collected either before or at least 2 h after meals, and all subjects abstained from smoking, eating or drinking for 2 h before their examination. UWS was collected for 10 min by the spitting method. After a 5-min rest period, RS was collected from the buccal mucosa on both sides of the mouth. Subjects were asked to swallow just before the RS was collected. Collection was done by gentle scraping of the buccal mucosa with a metal spatula. SWS was then collected for 5 min by chewing 1 g of gum base, after discarding the saliva collected during the first 2 min. UWS and SWS samples were collected into chilled sterile tubes, and the collection period was so timed that a flow rate (ml min^{-1}) could be calculated. RS samples were collected by pipetting the saliva on the metal spatula. All saliva samples were centrifuged at 3500 *g* for 20 min at 4°C; the resulting clarified supernatant fluids were analysed in our experiments.

Determination of total protein concentration
Total protein concentration in the saliva samples was determined by the bicinchoninic acid assay (Smith *et al*, 1985) with QuantiPro kit (Sigma, St Louis, MO, USA) using bovine serum albumin as the standard.

Determination of total carbohydrate and sialic acid concentration
Total carbohydrate in the saliva samples was determined using the phenol/sulphuric acid method with glucose as the standard (Dubois *et al*, 1956). Sialic acid content of the saliva samples was determined by the thiobarbituric acid assay (Warren, 1959).

Determination of sIgA and lactoferrin concentration
Salivary sIgA concentration was determined using an indirect competitive enzyme immunoassay kit (Salimetrics, State College, PA, USA) developed for saliva samples. Salivary lactoferrin concentration was determined using an avidin–biotin enzyme immunoassay kit (Calbiochem, Darmstadt, Germany) using lactoferrin from human milk as the standard.

Determination of peroxidase, lysozyme and α-amylase activity
Peroxidase activity was measured by the rate of oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5′-dithiobis(2-nitrobenzoic acid) $(Nbs)_2$ by $OSCN^-$ ions generated during the oxidation of SCN^- by salivary peroxidase (Mansson-Rahemtulla *et al*, 1986). Salivary lysozyme activity was determined with the Enzchek lysozyme assay kit (Molecular Probe, Eugene, OR, USA). Saliva samples were incubated with the substrate, *Micrococcus lysodeikticus* labelled with fluorescein. The fluorescence was measured in a FLUO Star Optima fluorescence microplate reader (BMG Labtech, Offenburg, Germany) using excitation/emission wavelengths of 485/520 nm respectively and the lysozyme activity was calculated from a standard curve prepared using lysozyme from chicken egg white. Salivary α-amylase activity was analysed by a salivary α-amylase assay kit (Salimetrics) which employs a chromogenic substrate, 2-chloro-*p*-nitrophenol linked to maltotriose.

Statistical analysis
A paired *t*-test was used to compare the salivary composition among RS, UWS and SWS. Correlation analysis was used to examine the relationships between flow rates (UWS and SWS) or amounts (RS), and composition or activity of each component in the RS.

Results

The mean flow rates of UWS and SWS were 0.46 ± 0.24 and 0.95 ± 0.45 ml min^{-1}, respectively. The amount of RS obtained each time by the scraping method was 91.3 ± 16.2 μl. The RS showed much higher concentrations of total protein, sIgA, lactoferrin, total carbohydrate and sialic acid ($P < 0.01$) than UWS and SWS. Furthermore, the enzymatic activity of peroxidase in the RS was higher than that of UWS and SWS ($P < 0.001$) (Table 1).

By dividing the concentration or enzymatic activity of each component by total protein concentration (total carbohydrate in the case of sialic acid), the relative proportion or specific enzymatic activity of each component was calculated. Compared with the UWS and SWS, the RS had a higher proportion of carbohydrate ($P < 0.01$) and lactoferrin ($P < 0.001$) and lower specific activity of lysozyme ($P < 0.001$) (Table 2).

The flow rates of UWS and SWS did not affect the concentration or enzymatic activity of each component in the RS. The relative proportion or specific enzymatic activity of each component in the RS was not affected by the flow rate of UWS and SWS, either. The amount of RS was negatively correlated with its lysozyme activty ($r = -0.456$, $P < 0.05$) and specific activity ($r = -0.397$, $P < 0.05$) (Tables 3 and 4).

Table 1 Concentrations of total protein, total carbohydrate, sIgA, lactoferrin and sialic acid, and enzymatic activities of peroxidase, lysozyme and α-amylase in residual, unstimulated and stimulated whole saliva samples ($n = 30$)

	UWS		*SWS*		*RS*		*P*-value[a]		
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*UWS:SWS*	*UWS:RS*	*SWS:RS*
Concentration (μg ml^{-1})									
Total protein	785.6	383.2	646.2	272.3	1784.0	1000.9	0.001***	0.000***	0.000***
Total carbohydrate	26.3	13.1	23.2	9.2	117.7	64.6	0.193	0.000***	0.000***
sIgA	173.2	93.9	107.6	59.5	282.3	208.3	0.003**	0.005**	0.000***
Lactoferrin	1.3	0.6	1.0	0.3	10.5	4.5	0.001***	0.000***	0.000***
Sialic acid	4.6	2.3	4.4	3.7	27.6	16.7	0.681	0.000***	0.000***
Enzymatic activity (U ml^{-1})									
Peroxidase	0.012	0.005	0.011	0.004	0.035	0.022	0.501	0.000***	0.000***
Lysozyme	1603.1	797.7	728.7	508.8	1144.4	936.3	0.000***	0.028*	0.027*
α-Amylase	12.6	11.8	19.7	22.6	24.7	32.9	0.098	0.025*	0.408

UWS, unstimulated whole saliva; SWS, stimulated whole saliva; RS, residual saliva.
[a]Paired *t*-test statistics for mean comparison.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2 Relative proportions of total carbohydrate, sIgA, lactoferrin and sialic acid, and specific activities of peroxidase, lysozyme and α-amylase in residual, unstimulated and stimulated whole saliva samples ($n = 30$)

	UWS		*SWS*		*RS*		*P*-value[a]		
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*UWS:SWS*	*UWS:RS*	*SWS:RS*
Concentration/total protein concentration (relative proportion)									
Total carbohydrate	0.04	0.02	0.04	0.01	0.06	0.03	0.954	0.005**	0.001**
sIgA	0.24	0.15	0.21	0.19	0.21	0.12	0.430	0.333	0.926
Lactoferrin ($\times 10^{-2}$)	0.20	0.07	0.17	0.06	0.57	0.33	0.073	0.000***	0.000***
Concentration/total carbohydrate concentration (relative proportion)									
Sialic acid	0.23	0.17	0.23	0.25	0.35	0.37	0.956	0.125	0.054
Specific activity (enzymatic activity/total protein concentration) (mU μg^{-1})									
Peroxidase	0.018	0.011	0.019	0.009	0.017	0.007	0.699	0.624	0.260
Lysozyme	2620.9	1937.7	1250.1	970.0	525.9	357.1	0.000***	0.000***	0.000***
α-Amylase	16.1	14.2	30.8	32.4	19.4	23.7	0.022*	0.450	0.053

UWS, unstimulated whole saliva; SWS, stimulated whole saliva; RS, residual saliva.
[a]Paired *t*-test statistics for mean comparison.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3 Correlations between flow rate (whole saliva) or amount (residual saliva) and concentration or enzymatic activity of each component in residual saliva ($n = 30$)

	UWS (ml min^{-1})		*SWS (ml min^{-1})*		*RS (μl)*	
	0.46 ± 0.24		*0.95 ± 0.45*		*91.3 ± 16.2*	
	r^a	*P*-value	r^a	*P*-value	r^a	*P*-value
Concentration in residual saliva						
Total protein	−0.109	0.406	0.020	0.879	−0.206	0.114
Total carbohydrate	−0.112	0.555	0.147	0.438	−0.276	0.139
sIgA	−0.134	0.481	−0.154	0.417	−0.297	0.111
Lactoferrin	−0.154	0.418	−0.085	0.655	−0.038	0.843
Sialic acid	0.240	0.202	0.037	0.848	0.340	0.066
Enzymatic activity in residual saliva						
Peroxidase	−0.130	0.492	−0.073	0.700	−0.283	0.129
Lysozyme	−0.287	0.125	−0.181	0.340	−0.456	0.011*
α-Amylase	−0.084	0.659	−0.075	0.694	−0.187	0.323

UWS, unstimulated whole saliva; SWS, stimulated whole saliva; RS, residual saliva.
[a]Pearson's correlation coefficient.
*$P < 0.05$.

Discussion

Dry mouth symptom is not always related to reductions in whole saliva output. Insufficient mucosal wetting and changes in salivary composition have been implicated as factors that influence the perception of dry mouth (DiSabato-Mordarski and Kleinberg, 1996; Wolff and

Table 4 Correlations between flow rate (whole saliva) or amount (residual saliva) and relative proportion or specific enzymatic activity of each component in residual saliva ($n = 30$)

	UWS (ml min^{-1})		*SWS (ml min^{-1})*		*RS (μl)*	
	0.46 ± 0.24		*0.95 ± 0.45*		*91.3 ± 16.2*	
	r^a	*P*-value	r^a	*P*-value	r^a	*P*-value
Concentration/total protein concentration (relative proportion)						
Total carbohydrate	0.120	0.529	0.348	0.059	0.160	0.400
sIgA	−0.085	0.656	−0.179	0.343	−0.336	0.069
Lactoferrin	0.092	0.630	0.030	0.877	0.328	0.077
Concentration/total carbohydrate concentration (relative proportion)						
Sialic acid	0.186	0.326	−0.041	0.830	0.296	0.113
Specific activity (enzymatic activity/total protein concentration)						
Peroxidase	0.170	0.369	0.154	0.416	0.171	0.367
Lysozyme	−0.216	0.251	−0.108	0.571	−0.397	0.030*
α-Amylase	−0.087	0.648	−0.129	0.496	−0.179	0.343

UWS, unstimulated whole saliva; SWS, stimulated whole saliva; RS, residual saliva.
[a]Pearson's correlation coefficient.
*$P < 0.05$.

Kleinberg, 1998; Dawes, 2004). Therefore, it was suggested that the measurement of oral mucosal wetness could be used as one of the diagnostic modalities for assessing the dry mouth condition. The information on the composition as well as the amount of RS is needed to understand further the biological role of the RS in the oral cavity (Wolff and Kleinberg, 1998; Won *et al*, 2001; Kleinberg *et al*, 2002; Lee *et al*, 2002).

The present study showed that the concentrations of total protein, important antimicrobials, and carbohydrates including sialic acid are elevated in the RS compared with those of UWS and SWS. A high concentration of total protein in the RS has been previously reported and a negative correlation between the mucosal wetness and the protein concentration of RS suggests that increased protein concentration is caused by decreased residual volume (Won *et al*, 2001; Lee *et al*, 2002). The elevated carbohydrate to protein ratio in the RS suggests increased amounts of glycoproteins in the RS. In fact, many of the protective functions of saliva can be attributed to the physical, structural and rheological characteristics of salivary glycoproteins, such as mucins, proline-rich glycoproteins, α-amylase, lactoferrin, salivary peroxidase, sIgA, carbonic anhydrase, kallikrein and fibronectin (Levine *et al*, 1987; Cohen and Levine, 1989). Salivary mucins and proline-rich glycoproteins are major proteins in terms of carbohydrate content. Notably, most sialic acid-containing units are located in salivary mucins (Levine *et al*, 1987; Cohen and Levine, 1989) such that high sialic acid concentration in the RS can be regarded as the result of concentrated salivary mucins on the oral mucosal surface.

The influence of minor salivary gland secretions can be another explanation for the high concentration of total protein and carbohydrate in the RS. RS contains a greater proportion of minor salivary gland secretions compared with whole saliva, and minor salivary gland secretions contain large quantities of proteins and carbohydrate-containing glycoproteins for oral mucosal defence (Hensten-Pettersen, 1976; Won *et al*, 2001; Lee *et al*, 2002).

The concentrated mucins on the oral mucosal surface play an important role in the maintenance of oral health by presenting multiple host defence functions on its surface. Such functions include: (1) acting as a permeability barrier against environmental insult at the tissue–environmental interface; (2) lubricating oral surfaces; (3) concentrating antimicrobial molecules onto oral surfaces; and (4) modulating colonization of oral bacteria, fungi and viruses (Schenkels *et al*, 1996). This functional diversity of salivary mucins is predicted, in part, by the carbohydrate content of these molecules (Hatton *et al*, 1985). Water retention because of solvation of carbohydrate residues is an important physicochemical factor that contributes to the lubrication function of mucins. In the intertangled networks of mucins, oligosaccharide chains contribute to the viscosity not only by size, but also by intermolecular interactions (Reeh *et al*, 1990).

Non-covalent interactions or complexes of salivary mucins with other salivary molecules can be a mechanism whereby salivary proteins concentrate in the saliva–tissue interface on the oral mucosa in the present study. Salivary mucins have been reported to undergo non-covalent interactions with sIgA, lysozyme, cystatins, α-amylase, proline-rich proteins, statherins, histatins and lactoferrin (Biesbrock *et al*, 1991; Iontcheva *et al*, 1997; Soares *et al*, 2003). In this type of complexing, mucins may act as molecular chaperones by protecting other molecules from enzymatic degradation or by concentrating antimicrobial molecules to oral surfaces. It is possible that the complex of molecules may have additional functions beyond that of the individual molecules comprising the complex (Levine, 1993). For example, the binding of low-molecular-weight salivary mucin (MG2) to *Pseudomonas aeruginosa* and *Staphylococcus aureus* has been shown to occur because of its association with sIgA (Biesbrock *et al*, 1991). The present study shows that sIgA, lactoferrin and peroxidase are concentrated in the RS and these concentrated antimicrobials may play important roles for mucosal defence. The fact that relative proportion of lactoferrin and specific activity of lysozyme in the RS differed from

those of whole saliva, suggests that all components in whole saliva are not concentrated to the same degree in RS. The decreased specific activity of lysozyme in the RS could be due to a low relative proportion of lysozyme or inhibition of lysozyme activity by other concentrated components such as mucins.

The high carbohydrate levels in RS on the buccal mucosa could serve as a targeting site for the delivery of therapeutic agents. Examples include binding of tetracycline to sialic acid as well as the protein moieties of mucin (Braybrooks *et al*, 1975; Kearney and Marriott, 1982). Mucin gel network may serve as a weak cationic exchanger or act as a molecular sieve (Desai *et al*, 1991). Furthermore, mucous secretions can lead to decomposition or binding of penetrating compounds, such as hydrogen peroxide being degraded by the concentrated salivary peroxidase present in the mucin network making the mucosal pellicle and RS almost impermeable to hydrogen peroxide (Schenkels *et al*, 1996; this study).

In clinical situations where salivary flow is decreased or lacking, the protective barrier provided by the oral mucosal pellicle and RS is severely compromised or lost. A concurrent change in the composition of RS associated with decreased volume in hyposalivators may occur. To be effective, saliva substitutes may be needed to supplement the deficient components of mucosal pellicle and RS and restore the compromised protective barrier. Further studies on the composition of RS in hyposalivators may provide guidelines for designing such saliva substitutes.

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