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Determination of salivary levels of mucin and amylase in chronic periodontitis patients

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Background and Objective: Patients with periodontal disease show differences in the profile of proteins in whole saliva. This profile reflects the nature and amplitude of the host response to a periodontal microbial challenge. Since periodontitis is a chronic inflammatory disease with different progression stages, the aim of the study was to evaluate the host response in these different clinical stages by assessing salivary flow rate, the concentrations of proteins and mucin and the amylase activity.

Material and Methods: Sixty adult subjects were clinically examined and distributed into four groups ($n = 15$) according to the periodontal status, namely, healthy, mild, moderate and severe periodontitis. Whole saliva was collected for 5 min, followed by a second 5 min sampling period with stimulation by chewing a paraffin block, and flow rate was determined. Salivary proteins, amylase and mucin were determined by colorimetric methods.

Results: The concentrations of proteins, amylase and mucin increased in subjects with moderate and severe periodontal disease in unstimulated saliva, while flow rate decreased. A positive correlation was found between proteins and amylase or mucin concentrations among the different groups, indicating that the concentrations changed in the same way, being the response of salivary glands to the disease, possibly to enhance the protective potential of saliva. Mucin concentration was lower in the mild periodontitis group. Mechanical stimulation induced an increase in flow rate and output of proteins, amylase and mucin.

Conclusion: Periodontitis induces an increase in the output of proteins, including mucin and amylase, thereby enhancing the protective potential of saliva, but this is accompanied by a decrease in flow rate.

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Human saliva is a body fluid secreted by major and minor salivary glands which is essential for the health of the oral cavity. Saliva contains a complex mixture of proteins with different biological roles in digestion, host defense and lubrication (1). Salivary immunoglobulins and mucins are believed to support clearance of microorganisms from the oral cavity. Salivary mucins are heavily glycosylated high-molecular weight glycoproteins produced by submandibular, sublingual and palatal glands and the minor salivary glands in the lip, cheek and tongue. Mucins play a major role in the maintenance of the viscoelastic properties of saliva, participate in formation of the protective oral mucosal mucus coat and tooth enamel pellicle (2), and promote bacterial aggregation and clearance from

the oral cavity (3). Human saliva has been shown to contain two structurally and functionally distinct populations of mucins, the high-molecular weight MG1 and the low-molecular weight MG2 mucins. MG1 coats and lubricates oral surfaces, while MG2 interacts with bacteria. Amylase is an abundant salivary component that catalyses the hydrolysis of α(1,4)glycosidic bonding between glucose residues of polysaccharides such as starch, glycogen and dextrins. In addition to saliva, amylase has been demonstrated in virtually every mucosal fluid of the human body, such as tears, semen and bronchial mucus (4). This ubiquitous distribution of amylase is difficult to reconcile with its single function of digestion. It would thus be appropriate to seek other functions for amylase in line with the concept of multifunctional saliva proteins (5). In this respect, it has been reported that amylase displays inhibitory activity against microorganisms (6,7).

Salivary gland secretion is a nerve-mediated reflex. Amylase and mucin are released by exocytosis from parotid and submandibular glands respectively in response to β-adrenergic stimulation. Muscarinic cholinergic stimulation evokes most of the salivary fluid secretion but causes a variable degree of exocytosis (8).

Periodontal diseases are highly prevalent chronic infections caused by a specific group of gram-negative anaerobic bacteria, leading to inflammation of the gingiva and destruction of periodontal tissues. Patients with periodontal disease have differences in the protein composition of whole saliva (9).

Chronic periodontitis can be classified into three stages on the basis of clinical parameters and the severity of the periodontal tissue destruction (10). The composition of saliva reflects the nature and amplitude of the host response to a periodontal microbial challenge (11). The aim of the study was to evaluate the salivary flow rate, proteins and mucin concentrations and amylase activity in stimulated and unstimulated whole saliva in different stages of periodontal disease compared with healthy periodontal conditions.

Material and methods

Subjects

For this study, adult subjects, the parents of children in a preventive dental programme, who were not receiving dental care and were not previously periodontally diagnosed, were randomly recruited at a private dental clinic. Before the study, the health of the subjects was ascertained by using detailed medical histories and clinical examinations, which confirmed that they did not use any medicine and were not smokers. Subjects gave their informed consent, and the assessment of clinical parameters was carried out by a calibrated dentist (G.S.; calibrated by the Argentine Dental Association and the University of Buenos Aires) including probing pocket depth (measurements were rounded off to the nearest millimeter marking), clinical attachment level (measuring the distance from the cemento-enamel junction to the bottom of the probable pocket) and bleeding on probing (scored as: −, no bleeding or +, bleeding within 30 s after probing). All periodontal disease measurements were performed in four quadrants using a first-generation probe (Hu-Friedy Mfg. Co., Chicago, IL, USA). Probing pocket depth and clinical attachment level were assessed at six sites per tooth and bleeding on probing at four sites per tooth. Subjects were distributed into four groups according to their clinical attachment loss, probing pocket depth and bleeding on probing (10), namely, healthy, mild, moderate and severe periodontitis (Table 1). Fifteen subjects were recruited for each group, with one female in the healthy and mild groups, none in the moderate group and five in the severe group. The protocol was approved by the Ethics Committee of the School of Dentistry, University of Buenos Aires, and the study was conducted in accordance with the Declaration of Helsinki 1975, as revised in 2000.

Collection of saliva

Whole saliva was collected by spitting into an ice-cooled graduated vessel. Subjects spat every 30 s for 5 min. Next, stimulated whole saliva was obtained after chewing paraffin for 5 min. The volume of saliva was recorded and expressed as millilitres per minute. The resulting saliva was stored in aliquots at −20°C until determinations were performed. All examinations were performed at 10.00 h by the same examiner.

Determination of protein, amylase and mucin concentrations

Colorimetric methods were used for all determinations in both stimulated and unstimulated saliva. The protein concentration was determined the Lowry method (12), and the amylase activity was determined in diluted saliva (1:100) by the method described by Bernfeld (13), using starch suspension as the substrate. Amylase activity is expressed as units per milligram wet weight, where 1 U amylase was defined as the quantity of enzyme that liberates 1 mg of maltose in 1 min at 20°C. Mucin concentration was determined using the Alcian Blue method described by Hall *et al.* (14) and modified by Sarosiek *et al.* (15). Briefly, aliquots of diluted saliva (1:10 dilution) were incubated for 30 min in a 1% solution of Alcian Blue in 50 mM sodium acetate buffer containing 25 mM $MgCl_2$, pH 5.8, under constant agitation at room temperature. Following incubation, the samples were centrifuged for 20 min at 705 *g*. The pellets were washed in 95% ethanol, vortexed gently for 10 s and, after 5 min, centrifuged for 20 min at 3000 rpm. Mucin–dye complexes were dissociated by the addition of a 1:2 dilution of Aerosol OT (Sigma Chemical Co., St Louis, MO, USA) in distilled water, brief mixing and sonication. Subsequently, samples were extracted with equal volumes of ethyl ether under vigorous shaking. The resulting solution was centrifuged for 15 min at 3000 rpm and the dye concentration determined spectrophotometrically at 605 nm in the aqueous layer. This method did not allow distinguishing between larger mucin, MG1, and smaller, MG2. The output per minute of proteins, amylase and

Table 1. Age, sex and number of sites in each patient with clinical attachment loss ≥ 4 mm, probing pocket depth ≥ 5 mm and bleeding on probing (+ or −), grouped according to Page & Eke (10)

	Healthy					Mild periodontitis					Moderate periodontitis					Severe periodontitis				
No.	Age	Sex	Clinical attachment loss	Probing pocket depth	Bleeding on probing	Age	Sex	Clinical attachment loss	Probing pocket depth	Bleeding on probing	Age	Sex	Clinical attachment loss	Probing pocket depth	Bleeding on probing	Age	Sex	Clinical attachment loss	Probing pocket depth	Bleeding on probing
1	33	M	0	0	−	46	M	0	0	+	54	M	2	1	+	40	M	6	3	+
2	38	M	0	0	−	41	F	0	0	+	48	M	0	3	+	61	F	4	4	+
3	33	M	0	0	−	40	M	0	0	+	57	M	1	2	+	48	F	5	3	+
4	41	M	0	1	−	37	M	0	1	+	42	M	3	0	+	46	M	7	2	+
5	35	M	0	0	−	32	M	0	0	+	43	M	2	0	+	35	F	6	3	+
6	37	M	1	0	−	42	M	1	0	+	47	M	1	3	+	32	F	5	4	+
7	40	M	0	1	−	38	M	0	1	+	41	M	3	0	+	44	F	4	4	+
8	34	M	0	0	−	45	M	0	1	+	38	M	3	0	+	47	M	4	3	+
9	36	M	0	0	−	33	M	0	0	+	40	M	1	2	+	48	M	3	3	+
10	36	M	0	0	−	35	M	0	0	+	43	M	2	1	+	45	M	5	5	+
11	35	F	0	0	−	37	M	1	0	+	36	M	2	0	+	40	M	6	3	+
12	33	M	0	0	−	41	M	0	1	+	37	M	1	2	+	43	M	4	2	+
13	32	M	0	0	−	38	M	0	0	+	35	M	1	3	+	55	M	7	3	+
14	30	M	0	0	−	30	M	0	0	+	38	M	1	3	+	50	M	5	3	+
15	27	M	0	0	−	36	M	0	0	+	36	M	2	0	+	53	M	6	4	+

The number in the first column refers to the number of each patient belonging to each group. M, male; F, female.

mucin was calculated based on the salivary flow rate (in milliliters per minute) of each patient.

Statistical analysis

Statistical significance of differences was determined by analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test. Student's paired *t*-test was used to compare stimulated and unstimulated saliva parameters from the same subject. Correlations were done using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). Differences between means were considered significant at $p < 0.05$.

Results

Clinical evaluation

Table 1 shows the population age, sex and the number of sites in each patient with clinical attachment loss ≥ 4 mm, probing pocket depth ≥ 5 mm and bleeding on probing (+ or −), grouped according to Page & Eke (10). Probing pocket depth score (mean ± SEM) was 1.9 ± 0.12 mm for healthy subjects, 2.2 ± 0.08 mm ($p < 0.05$) for mild, 2.6 ± 0.06 mm ($p < 0.001$) for moderate and 3.4 ± 0.1 mm ($p < 0.001$) for severe periodontitis. Clinical attachment level score was 0 for healthy subjects, 0.1 ± 0.03 mm ($p < 0.05$) for mild, 0.3 ± 0.03 mm ($p < 0.001$) for moderate and 0.5 ± 0.04 mm ($p < 0.001$) for severe periodontitis. The age range for healthy subjects was 27–41 years, for mild periodontitis 30–46 years, for moderate periodontitis 35–57 years and for severe periodontitis 32–61 years. Differences in salivary parameters were not caused by the age differences, since the eldest subjects in the periodontitis groups displayed comparable values to the younger subjects.

Unstimulated salivary parameters

The unstimulated salivary flow rate, concentrations of total proteins and mucin, and amylase activity in healthy subjects and in patients with mild, moderate and severe periodontal disease were determined (Table 2). While the salivary flow rate was significantly lower in patients with severe periodontitis, the total protein and mucin concentration and the amylase activity were significantly higher in patients with moderate and severe disease. Conversely, in the mild periodontitis group, mucin concentration was significantly lower than in healthy subjects. When amylase and mucin were expressed as units or milligrams per milligram of protein, the same results were obtained. It is noteworthy that protein and mucin concentrations, as well as amylase activity, increased from mild to severe periodontitis.

Correlation tests

Figure 1 shows the correlation between unstimulated salivary flow rate, protein and mucin concentrations and amylase activity belonging to all groups. No correlation between unstimulated flow rate and proteins or amylase was observed, but a significant inverse correlation ($r = 0.4055$, $p < 0.01$) was found between flow rate and mucin concentration (Fig. 1A–C). A significant correlation was detected between proteins and both amylase and mucin ($r = 0.3650, p < 0.01$ and $r = 0.4528$, $p < 0.01$, respectively) and between amylase and mucin ($r = 0.3267$, $p < 0.05$; Fig. 1D–F). Correlations were performed only for unstimulated saliva samples because the objective was to evaluate whether a decrease in unstimulated flow rate was responsible for the increase in protein concentration and whether mucin and amylase in unstimulated conditions changed in the same way in the different stages of the disease.

Stimulated salivary parameters

Table 3 shows the flow rate and proteins and mucin concentrations and the amylase activity in mechanically stimulated saliva. In patients with moderate and severe disease, the flow rate after mechanical stimulation was significantly lower than in the healthy group, while a significant increase in the concentration of proteins was observed. While no differences were observed in salivary amylase activity among groups, mucin concentration was significantly lower in the mild periodontitis group.

Comparison between salivary flow rate and output of proteins, amylase and mucin before and after mechanical stimulation

In Fig. 2 the flow rate and the output of proteins, amylase and mucin obtained before and after stimulation are shown. Stimulation induced a significant increase in flow rate in all groups, but in the severe periodontitis group the increment was significantly less (Fig. 2A). The output of proteins increased significantly in all groups after stimulation (Fig. 2B), while amylase and mucin increased only in the healthy and mild periodontitis groups (Fig. 2C,D). In the moderate and severe periodontitis groups, the output of mucin and amylase in

Table 2. Mean ± SEM values of salivary flow rate, concentrations of total proteins and mucin, and amylase activity in unstimulated whole saliva from healthy subjects and from subjects with mild, moderate and severe periodontal disease

Group	Flow rate (mL/min)	Proteins (mg/mL)	Amylase (U/mL)	Amylase (U/mg protein)	Mucin (mg/mL)	Mucin (mg/mg protein)
Health	0.52 ± 0.012	3.99 ± 0.13	89.63 ± 11.0	22.6 ± 2.8	1.9 ± 0.12	0.48 ± 0.04
Mild	0.48 ± 0.016	3.76 ± 0.17	87.55 ± 3.0	24.0 ± 1.5	1.4 ± 0.14**	0.38 ± 0.04
Moderate	0.48 ± 0.016	4.56 ± 0.14*	122.52 ± 6.8**	26.8 ± 1.3	2.7 ± 0.11***	0.61 ± 0.05*
Severe	0.43 ± 0.015*	4.65 ± 0.17*	136.94 ± 11.2***	30.0 ± 2.5	3.1 ± 0.06***	0.69 ± 0.04**

Significant difference from healthy subjects: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

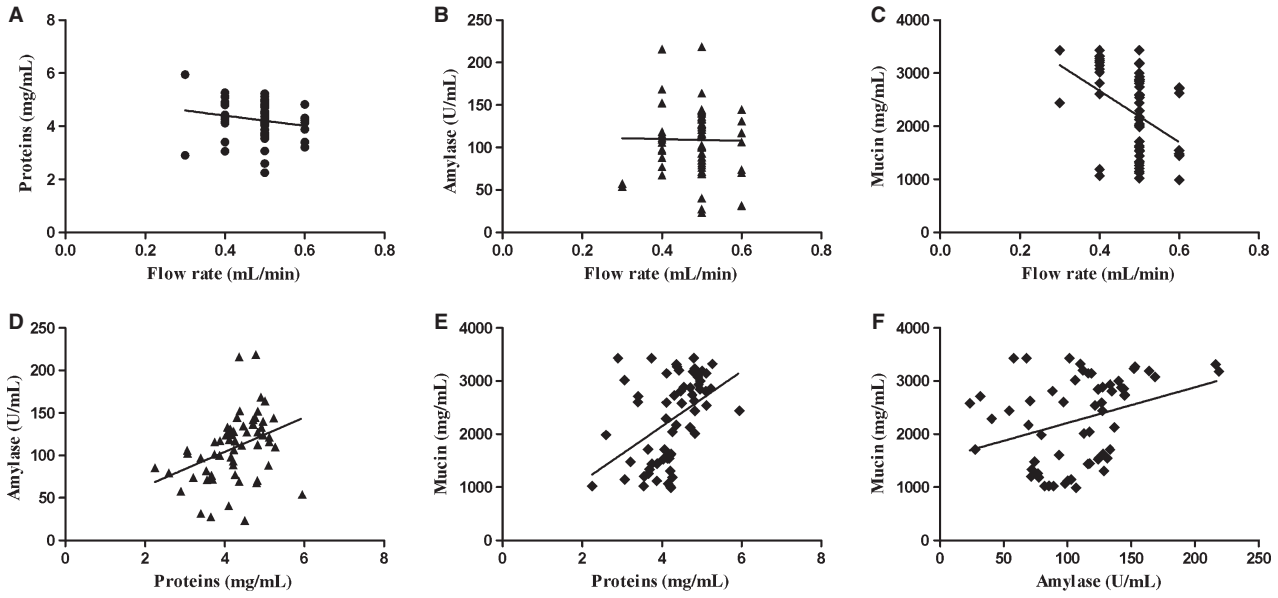

Fig. 1. Correlation between flow rate, proteins, amylase and mucin in unstimulated saliva from all of the subjects studied. Top panels show flow rate plotted as a function of proteins (A), amylase (B) or mucin (C). Bottom panels show protein plotted as a function of either amylase (D) or mucin (E) and amylase plotted as a function of mucin (F). Each symbol represents one subject.

Table 3. Mean ± SEM values of salivary flow rate, concentrations of total proteins and mucin, and amylase activity in stimulated whole saliva from healthy subjects and from subjects with mild, moderate and severe periodontal disease

Group	Flow rate (mL/min)	Proteins (mg/mL)	Amylase (U/mL)	Amylase (U/mg protein)	Mucin (mg/mL)	Mucin (mg/mg protein)
Health	1.03 ± 0.016	3.17 ± 0.13	54.02 ± 9.8	16.8 ± 2.8	1.20 ± 0.16	0.61 ± 0.06
Mild	0.99 ± 0.033	2.23 ± 0.17	52.62 ± 2.9	25.4 ± 2.5	0.92 ± 0.05*	0.68 ± 0.09
Moderate	0.94 ± 0.025*	3.64 ± 0.14*	65.19 ± 6.8	18.7 ± 2.1	1.36 ± 0.07	0.78 ± 0.08
Severe	0.85 ± 0.024***	4.04 ± 0.18***	79.28 ± 10.4	19.5 ± 2.4	1.50 ± 0.06	0.81 ± 0.07

Significant difference from healthy subjects: *$p < 0.05$ and ***$p < 0.001$.

unstimulated conditions was significantly higher than in health ($p < 0.001$ for mucin and $p < 0.05$ for amylase), while no differences were observed in stimulated conditions (Fig. 2).

Discussion

In this study, we examined salivary flow rate, the concentrations of proteins and mucin and the amylase activity related to the progression of chronic periodontitis in adult individuals, in stimulated and unstimulated saliva, to further understand their evolution during the course of the disease. Each group consisted of 15 subjects, with one female in the healthy and mild periodontitis groups, none in the moderate periodontitis group and five in the severe periodontitis group. The almost total absence of females in the study was due to their reluctance to participate. The study was carried out in whole saliva because the aim was to evaluate amylase and mucin concentrations, and they are produced by the parotid and submandibular glands respectively.

Unstimulated flow rate was only significantly lower in the severe periodontitis group. In other studies, where flow rate was not evaluated according to the periodontal status, differences were not observed between healthy subjects and subjects with periodontitis (16). Salivary glands provide a resting flow of saliva into the mouth that fulfils the defense mechanism (4) and, according to the results obtained in periodontitis subjects, this flow is maintained until periodontitis becomes severe. Thus, a reduction in flow rate is not involved in the early stages of periodontal disease, but when gingival tissues destruction is advanced it can be altered.

The concentration of total proteins and mucin and the amylase activity were increased in unstimulated saliva from subjects with moderate and severe periodontitis. This increase in proteins and amylase cannot be attributed to a change in flow rate because no positive correlation was found. However, an inverse correlation was found between flow rate and mucin concentration in saliva, indicating that the increase in mucin is accompanied by a decrease in flow rate. This is apparent, because in the mild periodontitis group mucin was reduced without a change in flow rate and, conversely, in the severe periodontitis group mucin was increased and flow rate decreased. In fact, a real increase

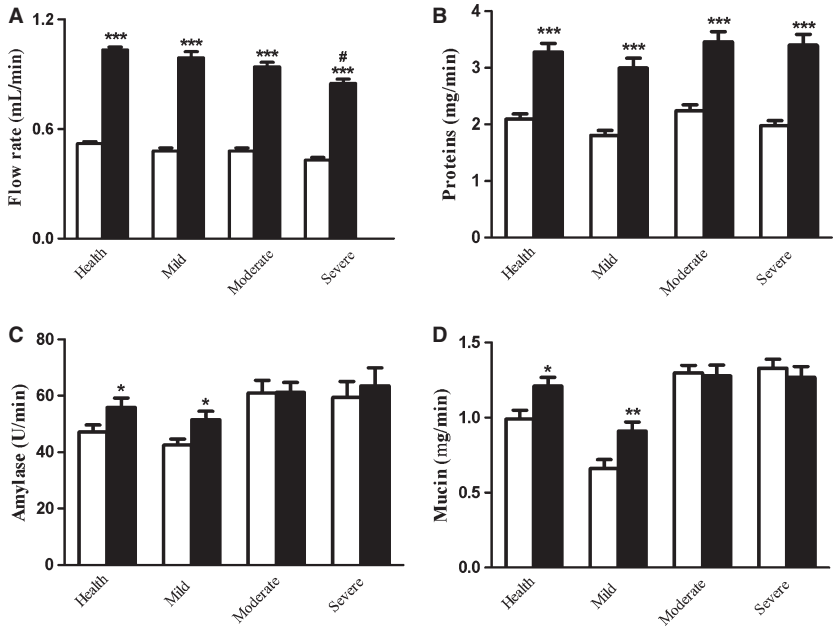

Fig. 2. Comparison of flow rate (A), output of proteins (B), amylase (C) and mucin (D) between unstimulated (open bars) and stimulated saliva (filled bars) from healthy subjects and patients with mild, moderate and severe periodontitis. The output of proteins, amylase and mucin was calculated based on the salivary flow rate of each patient. The bars represent the means ± SEM of 15 subjects per group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. unstimulated saliva from the same group. $p < 0.05$ vs. flow rate increment in all the other groups.

in mucin secretion occurred in the moderate and severe groups as derived from the output determinations. Salivary proteins have different biological roles in digestion, host defense and lubrication (1). The significant positive correlation observed among proteins, mucin and amylase and the fact that their salivary concentrations increased in relation to the evolution of the disease supports the view that they are implicated in the host response. Mucin is one of the proteins that protect the oral cavity against microbial infection (4), and amylase has been reported to increase in periodontal disease (16,17) and, in addition to saliva, it has been described in other mucosal fluids of the human body (4).

In the group where the inflammation reaction was mild, neither proteins nor amylase salivary concentrations were increased, but also mucin was decreased. This fact could be interpreted in the following two ways: first, the early stages of periodontal disease may induce a reduction in mucin secretion; or second, subjects with low mucin concentration are more susceptible to oral infections (18). In *Aggregatibacter actinomycetemcomitans*-infected patients, a decline in salivary MG2 concentration and output was found, and thereby, the susceptibility of the patients was enhanced (18). The study design was cross-sectional, not longitudinal, and for ethical reasons, after diagnosis the patients received appropriate periodontal care; therefore, the evolution of subjects with mild periodontitis could not be followed. Thus, the role of mucin concentrations as an inducing factor at the beginning of periodontitis could not be fully elucidated, but it is clear that the increase in mucin concentration in moderate and severe periodontitis may be considered as an attempt of salivary glands to enhance the protective potential of saliva.

Flow rate increased after mechanical stimulation in all groups, but after stimulation a lower flow rate was not only observed in severe periodontitis but also in the moderate disease, suggesting a lower response to the stimulus in this group. The increased flow rate induced the decrease in proteins and mucin concentrations and amylase activity in saliva, pointing out that exocytosis did not respond equally to the mechanical stimulation. (19). In spite of the dilution, differences in the concentration of proteins and mucin in the moderate and severe periodontitis groups were still observed. This can be related to the decrease in flow rate observed in these groups. Again, a decrease in mucin concentration was observed in the mild periodontitis group but, as stated above, the progression of the disease in this group could not be monitored for ethical reasons. It could be speculated that a decrease in mucin may be implicated in the pathogenesis of the disease, but it is a point that needs to be elucidated.

In order to evaluate the difference in exocytosis before and after mechanical stimulation, we calculated the output per minute of proteins, amylase and mucin. Output of proteins increased about 55–75% in all groups. Amylase and mucin increased only in the healthy and mild periodontitis groups, by about 20–35%. This fact can be explained, because mechanical stimulation may induce exocytosis of some proteins but little release of others (16). Unstimulated amylase and mucin output was higher in the moderate and severe periodontitis groups; thus, the lack of response to simulation could be related to depletion of acinar vesicles. The output of unstimulated mucin in the mild periodontitis group was the lowest. This fact was reflected in the salivary mucin concentration, but the response to stimulation showed that there was no impairment in salivary gland function. Thus, it can be assumed that the output of mucin increased in response to inflammatory stimuli as the disease progressed, having biological significance, as can be seen in the moderate and severe periodontitis groups. After stimulation there were no differences among groups, indicating that the salivary glands enhanced the protective potential of saliva in response to the inflammatory process during resting conditions.

We conclude that the development of periodontitis is not related to salivary gland dysfunction because, as the inflammatory disease progresses,

an increase in the concentrations of proteins, including mucin and amylase, is observed, which increases the protective potential of saliva. Exocytosis from salivary cells is related to sympathetic stimulation. The interaction between the sympathetic nervous system and the immune system during inflammation has been described (20). Thus, we can speculate that the infectious process of periodontitis activates the sympathetic system, which in turn leads to the release of some salivary proteins, thereby increasing the protective potential of saliva.

A different speculation may be done for the decrease in flow rate. It is known that in man, chewing on tasteless material results in an increase in parotid salivary flow, and the intraoral mechanoreceptors are involved in the masticatory salivary reflex (21). Thus, we propose that as periodontitis worsens and periodontal attachment loss increases, masticatory reflexes decrease and are responsible for the lower flow rate in the later stage of the disease.

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