Periodontal Status, Salivary Enzymes and Flow Rate in Passive Smokers

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Summary

The effect of passive smoking on prevalence and severity of periodontitis, saliva flow rate, total protein and activity of peroxidase and amylase was studied. In practice, 30 university students (18-22 years) who lived with smoker parents, friends or relatives considered as passive smokers and 30 healthy students in the same age range entered as control. A questionnaire was filled by each subject recording dental and dietary habits. Periodontal pocket depth was examined as a marker of periodontitis severity. Saliva flow rate, the concentration of total protein and biological activity of enzymes were measured on saliva samples. According to the results, periodontitis was either absent or had a pocket depth < 3mm in healthy subjects. Almost all passive smokers had one or two pockets with depth of ≥ 3mm. Salivary total protein showed slight alternation, salivary amylase activity and flow rate decreased while peroxidase activity increased in passive smokers. It was concluded that passive smoking was not only responsible for prevalence of periodontitis, but it also caused a decrease in saliva flow rate and salivary α-amylase activity as well as an increase in activity of peroxidase indicating incidence of oxidative stress.

Keywords: Periodontitis, amylase, peroxidase, total protein, passive smoking, saliva flow rate.

Introduction

An important report by World Health Organization (WHO) has indicated that passive smoking, especially among young children, may result in serious health hazards (1). According to this report, about 1 billion adults smoke worldwide and, therefore, it is estimated that at least 700 million children live in homes contaminated by tobacco smoke. This data may have been increased since 1999, especially in countries that smoking is not under serious governmental control. Having smaller bronchial tubes and less developed immune systems, children are the most susceptible group to suffer from the health hazards of passive smoking (2). Many investigators have studied the adverse effect of passive smoking on respiratory disorders such as asthma (3, 4), snoring or sleep fragmentation in children (5) and infants (6). It has been reported that the prevalence of childhood leukemia in children with smoker parents is also a symptom of passive (7). There are, however, a few literature indicating the relationship between passive smoking and general oral health or formation of dental caries in pre-school (8), primary school children (9, 10) and adolescent (11, 12). Periodontitis is a common infectious disease characterized by inflammation and destruction of periodontal tissue which can result in tooth loss.

Periodontal diseases are commonly diagnosed on the basis of clinical parameters such as periodontal probing pocket depth, bleeding of pockets and clinical attachment level (13). Salivary biomarkers could predict the presence and severity of periodontitis prior to the clinical evidences (14).

Human whole saliva is an important body fluid that contains a highly complex mixture of substances, similar in many aspects to other body fluids (15). It is secreted primarily by three paired major salivary glands and secondarily by hundreds of minor salivary glands located below the mucosal surfaces of the mouth (16, 17). Salivary gland secretions contain locally produced proteins, as well as other molecules from the systemic circulation. Variable amounts of blood, serum, serum products, gingival crevicular fluid (GCF), electrolytes, epithelial and immune cells, microorganisms, bronchial products and other foreign substances may also be found in whole saliva (18, 19). Therefore, salivary fluid could be used as a suitable biomarker for oral disorders (20, 21) as well as some

systemic disease (22). It has been shown that saliva can also reflect the relationship between oral hygiene and some chronic systemic diseases (23).

Salivary α-amylase is the first enzyme in the gastrointestinal tract for extracting caloric value from food. However, beyond the primary role of α-amylase to begin digestion of complex starches, sugars, and carbohydrates (24), salivary α-amylase is known be a surrogate marker of stress (25). It has also been found that salivary α-amylase may be influenced by behavioral and psychological factors and processes (26).

The peroxidase system of oral cavity is predominately involved in the high antioxidant capacity of saliva (27). This system is composed of two peroxidase enzymes, salivary peroxidase and myeloperoxidase. The salivary peroxidase secreted from the major salivary glands (28), contributes to 80% of oral peroxidase activity. On the other hand, myeloperoxidase, produced by leukocytes in inflammatory regions of the oral cavity (29), contributes to only 20% of oral peroxidase activity. The term oral peroxidase is used here to denote the total activity of both isoforms, since the 2-nitrobenzoic acid-thiocyanate (NBS-SCN) assay used is capable of measuring the activity of both enzymes.

Due to its ease of sampling, non invasive nature and reliable alternations of important biomarkers, saliva can be used as a valuable body fluid for diagnostic applications especially in children. We have previously reported alternations in activity of salivary peroxidase and α-amylase due to smoking (30, 31). The aim of this study was to investigate the association between passive smoking and periodontal status, salivary flow rate and activity of enzymes.

2. Materials and methods

A rapid colorometric direct α-amylase kit, Quantichrom α-amylase kit (DAMY-100), 4-amino antipyrine, phosphate buffer, hydrogen peroxide and phenol were obtained from Aria Orto Company, a local supplier of chemical, biological, medical reagents and kits.

Subjects

70 university students (aged 18-22 years old) were asked to fill out a questionnaire about the smoking habits and number of cigarettes smoked in their home per day. The subjects were then divided into passive smokers who lived in the smoking household (n=30, boys and girls) and non smokers, those who lived in homes with no smoking habits (n=30, boys and girls). 10 subjects, those with a friend or relative who rarely smoked, were excluded from the study. For ethical regulations, a complete explanation about our research, its objective, methods, anticipated benefits, and the inconvenience may the methodology bring about was given and a written informed consent for each subject's participation was obtained prior to sampling. A questionnaire about dental care and possible treatment as well as their dietary habits was also filled by each subject. All of the subjects had lived in Rasht for at least 2 years before the study where water fluoridation level is about 0.2 ppm. The exclusion criteria that were used for the sample also included the presence of systemic or dental disorders and the use of antibiotics or antimicrobial agents in the last 3 months.

Collection of saliva samples

Un-stimulated whole saliva samples were collected from all subjects one hour after breakfast. The volunteers were told to wash their mouth with about 5.0 ml of distilled water 2 times. After exactly 2 minutes, saliva specimen was collected into calibrated tubes, without exogenous stimulation. The collected samples were immediately centrifuged at 3000 rpm for 10 minutes at 4°C to remove cell derbies. They were then stored frozen until assay and the analysis was performed within 48 hours of collection.

Periodontal status

Periodontal probing pocket depth was conducted on both groups by a periodontist either prior to or after collection of saliva.

Saliva flow rate

Timed un-stimulated whole saliva samples (2 ml) were collected in clean, dry and sterile pre-weighted tubes. The flow rate was calculated by measuring the time required to collect one ml of saliva (ml/min).

Salivary total protein

All saliva samples were brought to room temperature slowly on the day of testing. They were then centrifuged again at 3000 rpm for 15 min, and the clear top phase of the sample was pipetted into appropriate test tubes. Salivary total protein was calculated by the method of Lowry *et al* (32). Bovine serum albumin samples (0.05-0.5 mg/ml) used as standards and were run simultaneously with the tests.

Salivary α-amylase activity

Centrifuged saliva samples were brought to room temperature and diluted by distilled water (1:4) before assay. The Quantichrom™ α-amylase kit was used for assessment of salivary α-amylase. The principle of kit was based on cleavage of an insoluble dye-coupled substrate, amylase azure into soluble colored product by the action of α-amylase. The intensity of produced color was measured at 595 nm and it was proportional to the activity of enzyme in the saliva samples (33). The biological activity of salivary amylase was the calculated using following equation:

$$\alpha\text{-amylase activity (U/L)} = \frac{OD_{sample} - OD_{blank}}{OD_{cal} - OD_{H2O}} \times n \times 550$$

Where OD_{sample}, OD_{blank}, OD_{cal} and OD_{H2O} are the optical density at 595 nm values of the sample, blank, calibrator and water respectively. The number "550" is the equivalent activity (U/L) of the calibrator under the assay conditions and "n" is the dilution factor. One unit of amylase activity was defined as the amount of enzyme that catalyzed production of one μmole of product per minute under assay conditions (pH 7.0).

Peroxidase activity in saliva

The biological activity of peroxidase on 4-amino antipyrine, its most common substrate, was measured spectrophotometrically as described before (31). In a typical assay, oxidation of 4-amino antipyrine was measured at 25°C in 3 ml of 0.3M phosphate buffer, pH 7.4, containing 0.0010 M hydrogen peroxide, 0.002 M 4-amino antipyrine and 0.15 M phenol. 40 ml of enzyme solution (6×10^{-4} mg/ml in 0.3 M phosphate buffer pH 7.4) was then added and the change in absorption at 510 nm (ΔA/min) was recorded. The change in absorption at 510 nm is due to the formation of a chromogen product with a λmax at 510 nm. One unit of activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under standard conditions.

Statistical analysis

The results for statistical analysis were compared between the passive smoking and non-smoking groups. Data on the activities of each group were observed and calculated, statistically. The two-sample Student *t*-test for differences in means was used to compare the means, and statistical significance was set at $p < .05$.

Results

Dietary and dental habits

Some important dietary and dental care habits of the participants in the research are presented in Table I. The differences in tooth-brushing habits, daily dietary sugar intake and exposure to smoke, between passive smoker and control subjects were not statistically significant ($p > 0.05$).

Table I. Dietary, smoking and dental care habits obtained from questionnaire.

Personal habit	No./day	Passive smokers (n=30)	Control subjects (n=30)
Sugar intake	<5	14 (46.6%)	15 (50%)
	≥5	16 (53.3%)	15 (50%)
Brushing	<2	20 (66.6%)	14 (46.6%)
	≥2	10 (33.3%)	16 (53.3%)
Flossing	0	4 (13.32%)	5 (16.66%)
	1	26 (86.68%)	25 (83.34%)
Gum bleeding	<2	20 (66.6%)	25 (83.34%)
	≥2	10 (33.3%)	5 (16.66%)
No. cigarettes smoked	<20	15 (50%)	--
	≥20	15 (50%)	--

Periodontal status

Probing pocket depth was measured as an indicator of periodontal status. The results are presented in Table II. Pocket depths of ≤ 3mm was considered as healthy and ≥ 6mm as severe periodontitis. It was observed that more than 90% of passive smokers suffered from moderate periodontal symptoms (pocket depths > 3 < 6mm).

TableII. Periodontal probing pocket depth in passive smokers compared to control group.

Pocket depth (mm)	Passive smokers (n=30)	Control subjects (n=30)
≤ 3	10 (33.3%)	28 (93.3%)
> 3 < 6	18 (60%)	2 (6.66%)
≥ 6	2 (6.66%)	0

Salivary flow rate, total protein, peroxidase and α-amylase

Salivary flow rate, total protein concentration, peroxidase activity and α-amylase activity are reported in Tables III-VI respectively. The flow rate was affected by smoking, i.e. a lower saliva rate was observed in passive smokers compared to control group. There was no detectable effect of passive smoking on total protein ($p > 0.05$). Oral peroxidase was found to be significantly inhibited in passive smokers compared to the control group. The activity of amylase in passive smoker group was found significantly higher compared with the controls ($p < 0.05$).

TableIII. Salivary Flow rate (ml/min) of passive smokers compared to control group.

Flow rate	Passive smokers (n=30)	Control subjects (n=30)
Mean	0.75	1.02
Range	0.55-0.97	0.78-1.25
Standard deviation (SD)	0.53	0.79

TableIV. Salivary total Protein (mg/ml) of passive smokers and control group

Total protein (mg/ml)	Passive smokers (n=30)	Control subjects (n=30)
Mean	1.44	1.62
Range	1.04-3.45	1.22-3.67
Standard deviation (SD)	0.78	0.89

Table V. Activity of salivary α-amylase in passive smokers compared to control group.

Amylase activity (U/ml)	Passive smokers (n=30)	Control subjects (n=30)
Mean	11.10	14.66
Range	7.42-12.66	9.88-25.2
Standard deviation (SD)	8.14	10.22

Table VI. Activity of salivary peroxidase of passive smokers and control subjects.

Peroxidase activity (U/ml)	Passive smokers (n=30)	Control subjects (n=30)
Mean	0.695	0.570
Range	0.201-1.812	0.194-1.025
Standard deviation (SD)	0.462	0.354

Discussion

According to the results obtained, it was shown that probing pocket depth of > 3 < 6 mm is about 10 times more prevalence in passive smokers compared to control subjects (Table II). However, severe periodontitis status (≥ 6 mm) was only observed in two passive smokers, while the control group was almost healthy periodontically and only two cases (6.66%) of 4 mm pocket depths were recorded by the dentist who examined all subjects. This type of results is in agreement with similar studies about the relationship between dental caries and smoking (11, 34). It has been demonstrated that formation of dental caries and plaque are closely related to the prevalence of periodontitis especially in smokers (34).

According to the results obtained from this research, moderate periodontitis is about twice more prevalent than healthy status in passive smokers, suggesting that passive smoking adversely affects gums and dental health in a way very similar to direct smoking (35-40). It can be explained by considering the fact that cigarette smoke is composed of two main phases: a tar phase and a gas phase; both of which are rich in various free radicals and non-radical oxidants. It has been estimated that a single cigarette puff contains approximately, 10^{14} free radicals in the tar phase, and 10^{15} radicals in the gas phase (41). These free radicals could initiate the generation of various reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$) and peroxy ($ROO^{\bullet}$) radicals. ROS are then capable of increasing and promoting oxidative damage in the form of lipid peroxidation and tissue damage leading to various stages of periodontitis (42).

Another important finding of this research was evidences of a decreased the salivary flow rate in passive smokers compared to the control group (Table III). The mean volume of saliva was decreased from 1.02 ml in healthy group to 0.75 ml in passive smokers indicating about 27% decrease in collected saliva volume per minute. This result in contrast with the results obtained for saliva flow rate due to smoking (43). However, they had examined stimulated saliva, while the saliva samples in this study were obtained without external stimulation. It is suggested that smoking may produce stimulated saliva of a relatively high pH which favors the mineralization of plaque in smokers leading to more persistent dental plaque.

Biochemical performance of saliva was also examined in this research in terms of total protein, biological activity of salivary peroxidase and α-amylase. According to the results, concentration of salivary total protein did not show significant variation in passive smokers compared to control (Table IV). A similar result was obtained for salivary protein concentration in school children with smoker parents (44).

On the other hand, activity of amylase was decreased in passive smokers compared to healthy group (Table V). Similar results have been reported by Granger et al who found lower salivary amylase activity for mothers, but not for infants as a result of exposure to tobacco smoke (44). However, variations in activity of the salivary amylase between passive smokers and control subjects were different in our study with those reported by Avşar et al (45) for younger children. The difference could be explained in terms of differences in age of subjects entered each investigation. In younger passive smokers, increased activity of amylase could be due to reduced amylase secretion due to the adverse effect of cigarette smoke. However, this is not important in the case of young adults for whom secretion of salivary factors are less affected by external factor. In a previous study, we had studied the effect of smoking on the activity of salivary amylase in university student with age range similar to this study. The results showed also a decrease in salivary amylase smokers as compared to non-smokers (30). It was explained that inhibition of salivary amylase by cigarette smoke may be due to the interaction between smoke aldehydes and –SH groups of the enzyme molecules. Moreover, the percentage of

the enzymatic inhibition showed a negative correlation with the basal level of salivary reduced gluthation (GSH). Regular exposure of passive smokers to cigarette smoke may accumulate smoke aldehydes in their saliva leading to their interaction with –SH group of amylase.

Salivary peroxidase showed a significant increase in passive smokers when compared to non-smoker group (Tables VI). This finding is similar to the result obtained for activity of peroxidase (31) and some other intercellular enzyme in saliva of smokers (46). It is known that the enzymatic antioxidant system plays an important part in oral defense mechanism, especially against the attack of free radicals related to smoke and, thus, prevents the evolution of oral cancer (47). Oral peroxidase is the most important part of salivary antioxidant system (48). Moreover, it is demonstrated that patients with oral lichen planus (a premalignant lesion) have a lower salivary antioxidant capability (49). It is known that cigarette smoke contains hydrogen cyanide, which is metabolized by the liver to SCN^- ranging 0.3-1.5 mM in normal saliva (31). In heavy smokers this range is increased to approximately 1.4–4.0 mM, depending on the number of cigarettes smoked per day, with a prolonged half-life of 9.5 h (48). Following SCN^- secretion via the saliva, it reacts with the H_2O_2 in the oral cavity, leading to elimination of the H_2O_2, a reaction catalyzed by oral peroxidase system. Therefore, increased activity of peroxidase could be due to nature response to elevation of SCN^- in saliva in one hand and higher concentrations of ROS created by cigarette smoke in the other. However, peroxidase is an intercellular enzyme that may become extracellular due to periodontal disorder in passive smokers leading to its secretion into the oral cavity.

Conclusions

Based on the results obtained from various sections of this research the following conclusions could be made:

1. Passive smoking is a serious risk factor in development of periodontal symptoms in young adults.

2. Passive smoking is associated with a decrease in salivary amylase activity and in young subjects.

3. Saliva flow rate is altered in young adult passive smokers, most of them experience about 25% reduction in their saliva flow rate.

4. Salivary total protein was not significantly altered due to passive smoking. It is believed that while some proteins are increased, the others show decreased concentration leading to non detectable variations in total protein concentration.

5. The effect of passive smoking on biological activity of salivary enzymes is different. Salivary amylase showed a decreased activity, but the activity of peroxidase was significantly increased.

Ethical approval

This research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version VI, 2002).

Conflict of interest

There was no conflict of interest and source of funding on this research.

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