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Research Article

To Study Amylase Levels in the Saliva of Khaini/Tobacco Chewers and Bidi Smokers with the Normal Controls

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Abstract

Tobacco consumption is regarded as one of the most important public health problem worldwide most importantly the development of oral cancers. It is consumed in diverse forms like smoking, chewing, snuff dipping, etc. in spite of being the major independent risk factor for the development of oral cancer. The use of saliva for diagnostic purpose is gaining wide momentum in recent years. It is considered as a reliable tool in hormone analysis, drug monitoring etc. Tobacco consumption has been reported to cause severe morphological and functional alterations in salivary glands. Based on these facts it could be postulated that tobacco use can adversely affect the salivary glands resulting in alteration in quality and quantity of saliva. Hence the present study is undertaken to evaluate the changes in the biochemical constituents of saliva in tobacco smokers and chewers and compare with those of healthy controls.

Materials and Method: Patients reporting to the OPD of the Department of Oral Pathology and Oral Medicine at Dr Z A Dental College, AMU Aligarh, were included in the study. The present study comprised of 150 subjects, 50 subjects had a habit of bidi (tobacco) smoking, 50 subjects had a habit of khaini (tobacco chewing) and 50 were gender and age related healthy subjects (with no smoking or chewing habit) i.e. the control group. All the subjects were male in the age group of 20 - 40 years as tobacco intake is more prevalent in the younger age group. Subjects were asked to refrain from eating, drinking or smoking one hour prior to collection of saliva. Each subject was asked to accumulate saliva in the mouth for about 2 minutes, after which he was asked to spit the accumulated saliva into a sterile plastic container. This was repeated for about 6 minutes for each subject and the unstimulated whole saliva thus collected was refrigerated at 4oC and processed within 24 hours for estimation of biochemical constituents.

Result: Salivary Amylase as calculated in smokers, chewers and controls was significantly reduced in both tobacco chewers and smokers. This could be attributed to the injury to Ductal Secretory unit caused by tobacco related toxic products or the effect of increase salivary flow with dilutional effect in tobacco users. The salivary flow rate in tobacco smokers is also found to be low. There is excess secretion of saliva in tobacco chewers as compared to smokers, as chewing may lead to hypertrophy of the masticatory muscles which may express greater salivary flow from the glands.

Keywords: Tobacco; PH; Saliva Smoking

Introduction

Saliva is an unique biological fluid and provides protection to the hard and soft tissues of the oral cavity with its cleansing, lubricating and anti-microbial properties. Oral mucosal integrity depends upon the normal salivary gland function. Therefore, it is accepted that salivary gland disease predisposes the oral mucosa to pathological alterations [1].

Tobacco is the greatest disease producing product known to man and also one of the most important public health problem worldwide including the development of oral cancer. Throughout the world, oral cancer occurs frequently in men than in women, with the male to female ratio greater than 2:1 [2]. Tobacco is consumed in various forms like smoking, chewing, snuff, etc. and is also the major independent risk factor for the development of oral and pharyngeal cancer and other malignancies of upper aero digestive tract [3].

The use of saliva for diagnostic purpose is fast gaining interest in research for hormone analysis, drug monitoring, alcohol and tobacco abuse and as a source of DNA for forensic applications. Studies have reported that there is a significant reduction of immunoglobulin A level in smokers due to combustion products of tobacco. Based on these facts tobacco use can have adverse effect on salivary glands resulting in change in quality and quantity of saliva [4]. The present study is undertaken to evaluate the changes in the biochemical constituents of saliva in tobacco smokers and chewers and compare with those of healthy controls. It is well known that saliva is a peculiar natural resource with many func-

tional abilities in digestion, lubrication and preparation of food, protection of the teeth and mucous membranes.

Salivary glands include the parotid, submandibular and sublingual glands. These are the major salivary glands. The parotid glands have serous acinar cells and produce a proteinaceous, watery secretion whereas secretion from the sublingual gland is mucous and hence more viscous. Submandibular glands have both serous and mucous acinar cells which reduce saliva with lower protein content and higher viscosity than parotid glands. Minor salivary glands are situated on the tongue, palate, buccal and labial mucosa. They are small mucosal glands with primarily mucous secretion. The main working part of the salivary gland tissue includes the secretory end pieces (acini) and the branched ductal system. The fluid first passes through the intercalated ducts which have low cuboidal epithelium and narrow lumen. Then the secretions enter the striated ducts which are lined by more columnar cells with many mitochondria. Finally the saliva passes through the excretory ducts where the cell type is cuboidal with stratified squamous epithelium [5].

Functions and composition of saliva

Functions of saliva can be organized into 5 major categories that serve to maintain oral health. 1) Lubrication 2) Buffering action 3) Maintenance of tooth integrity and protection 4) Antibacterial activity 5) Taste and digestion.

Composition of saliva consist of 99% water, 1% of large and small molecules and electrolytes. It's a well-recognized fact that it's a hypotonic fluid and not an ultra-filtrate of plasma. Hypotonic nature of saliva enables taste buds sense different tastes. Decreased levels of glucose, bicarbonate and urea in unstimulated saliva also manages the hypotonic environment to enhance taste.

Blood plasma products like Albumin, IgG, IgA, IgM, vitamins, drugs, hormones, water and ionic constituents are vital for proper functioning. Organic molecules like alpha amylase lipase histatins, cystatins epidermal growth factor, lactoferrin etc. are released by acinar cells. Duct cells are known to release lysozyme which plays important role in defence [6].

Organic Components

Main organic components are collection of proteins, alpha amylase lipase, immunoglobins etc.

Protein: Salivary proteins comprise approximately 200 mg/100 ml. It makes about 3% of the protein concentration in plasma.

Antibacterial Proteins include Lysozyme, Lactoferrin and Sialoperoxidase are antibacterial proteins.

Mucin: Mucin are high molecular weight glycoprotein released by mucus secreting cells. It lubricates oral mucosa and forms a barrier and prevents drying of the oral cavity.

Glycoproteins: Main groups of glycoproteins are Mucous glycoproteins present in submandibular and sublingual saliva and Proline rich glycoproteins (PRPs) in parotid products.

Other Polypeptides

Statherin and Sialin: These Phosphoproteins has a role in inhibiting hydroxyapatite crystal growth and utilizing several bacteria and forming alkaline end products.

Histidine Rich Peptide: It has a role in pellicle formation and bacterial clearance [7].

Alpha Amylase: Alpha amylase is a major digestive enzyme of saliva found in highest concentrations. It is involved in starch and polysaccharides metabolism It hydrolyses α1:4 glycosidic bonds between glucose units in the polysaccharide chain but very slowly in the terminal glucose units.

Lipase: Lipase is secreted by Von-Ebner's lingual salivary glands and is responsible for initial steps in fat digestion.

Immunoglobulins: Secretory IgA is the predominant immunoglobulin with IgG and IgM arising from the gingival crevice. It has direct action on oral bacteria and makes it difficult for the bacterial cells to bind to oral epithelium [8].

Inorganic Constituents

	Range	Mean
Sodium	0 - 80	15 resting 60 stimulated
Potassium	60 - 100	80
Calcium	2 - 11	6
Phosphorus (inorganic)	6 - 71	17 resting 12 stimulated
Chloride	50 - 100	-
Thiocyanate	-	9 (Smokers) 2 (Non-smokers)
Fluoride (Parts/106)	0.01 - 0.04	0.03 resting 0.01 stimulated
Bicarbonate	0 - 40	6 resting 36 stimulated
pH	5 - 8	-

Table 1: Inorganic constituents of whole saliva (MG/100ML).

Ions like Na^+, K^+, Cl^- and HCO^- plays very important role in the osmolality of saliva which equals half in relation to plasma. Bicarbonate are the main buffer with Fluoride content equals plasma and elevated in those where water supply exceeds fluoride limit etc. Fluorides importantly contribute to the anti-caries action of fluoride [7].

Salivary flow rate and factors

Normal range of unstimulated and stimulated salivary flow is 0.1 ml/min and 0.2 ml/min respectively. Due to of vast range of flow rates, it is difficult to assess the status of a patient's salivary

gland function from a single flow rate determination and hence repeated measurements are required to recognize, the declining flow rate and to prevent its deleterious consequences. On an average, unstimulated flow rate is 0.3 ml/min with 300 ml for 16 waking hours. Usage of standard technique with 1 minute of stimulation by chewing paraffin wax followed by salivary collection for 5 minutes, stimulated flow rate for adult female was 8.6 ml/5 mins, and adults males was 10.1 ml/5 mins [8]. On an average, stimulated flow rate is 7 ml/min and contributes 80 - 90% of average daily salivary production. Flow rate is 0 during sleep. The total daily salivary flow is 0.5 - 1.5 ml/min of whole saliva and flow rate less than 0.12 - 0.16 ml/min. is hypofunction [8].

Factors controlling flow rate

Diurnal Variation

Circadian variations plays vital role in the flow and the concentration level of components like electrolytes and proteins. Flow rate shows regional variation with mandibular lingual areas being high volume and maxillary anteriors being low [6].

A small amount of saliva on an average of about 0.8 ml that remains in the mouth. Its main role is in keeping oral tissues always moist [9].

Duration of stimulus

Flow rate influences the salivary composition. As flow rate increases, concentration of proteins, sodium chloride and bicarbonate levels rise and the levels of phosphate and magnesium fall [7-11]. For sodium and chloride, as the amount of primary secretion increases, the time during which the fluid is passing through the duct is reduced and thus the amount of ductal modification. Therefore at very high flow rates, the composition of the ductal fluid is approximate to that of primary acinar secretion. But the concentration of bicarbonate, calcium and protein begin to rise after some time of stimulation. Magnesium, phosphate and potassium concentration plateau after an initial fall. Calcium concentration decrease in times of active stimulants. After first few minutes of stimulation, sodium and iodide concentration remain unaffected [10].

Nature of stimulus

Flow rate is influenced by stimulus susceptibility of various glands and their proportionate secretions.

Dietary factors

Flow rate is directly and indirectly affected by gustatory and mechanical factors. For instance Copious salivary flow results from the smell of food or new denture insertion.

Hormonal influence

Aldosterone increases sodium reabsorption in striated ducts. Anti-diuretic hormone increases water reabsorption from the striated ducts. testosterone has positive effect on the salivary gland resulting in increased saliva secretion. Bradykinin and kallikrein increase salivary secretion reflecting increased acinar vasodilatation [10].

Salivary flow rate and systemic factors:

Ava J Wn., *et al.* (1993) showed statistically significant reduction in submandibular salivary flow rates in patients treated for one or more systemic disease when compared to that of parotid gland saliva flow rates exhibiting that the submandibular gland is more susceptible to pathologic and physiologic changes than parotid gland [11].

Tobacco

Tobacco addiction is a pandemic affecting the world with almost 1.3 billion smokers in the world. Number of death due to tobacco intake is around 5 million people a year with the same rate it is calculated that the number of deaths will nearly double, reaching close to 10 million by the year 2020. This trend is fast shifting to developing countries and the epidemic is expected to expand especially in low and middle income countries [12,13].

Contents of tobacco

Tobacco has an addictive potential containing substance which includes nicotine, carcinogens and other toxins. The addiction and exposure to high levels of tobacco are the root cause of all problems arising from tobacco usage. The diverse products in the plant, the poisons resulting from its processing and combustion are powerful and easily absorbed by many routes into the human body. Harmful contents of tobacco smoke are Hydrogen Cyanide, Ammonia, Toluene, Acetone, Methanol, Naphthalene, Carbon Monoxide, Vinyl Chloride, Dimethylnitrosamine, Arsenic, DDT, Urethane, Dibenzacridine, Pyrene, Cadmium, Benzopyrene and Naphthylamine [12].

Tobacco usage products

Tobacco comes in many forms and with various methods of use, with various names and claims attached to them. The different ways of usage include:

1. Tobacco rolled and smoked common examples are bidi, cigar, and cigarette)
2. Pipes which include traditional “hukka” and smokers pipe
3. Preparations in the mouth common example are chewing and placing in the mouth or keeping in the nose (E.g. snuff, betel quid and Gutkha) [12].

Effects of tobacco on health

Tobacco use is “the single most important preventable risk to human health in developed countries and an important cause of premature death worldwide [13]. Diseases linked to smoking tobacco cigarettes include, many forms of cancer, particularly lung

cancer, cancer of the kidney, cancer of the larynx, head and neck, breast cancer, bladder, esophagus, pancreas and stomach. There is some evidence suggesting an increased risk of myeloid leukemia, squamous cell sinonasal cancer, liver cancer, cervical cancer, colorectal cancer, childhood cancers, cancers of the gall bladder, adrenal gland and intestine, Cardiovascular diseases, blindness, cognitive dysfunction like increased risk of Alzheimer's disease, reduced memory Brain shrinkage, Impotence, etc. is also increased due to tobacco consumption [14].

Oral effects of tobacco

Diverse mucosal aberrations have been seen in long term users of smoked and smokeless tobacco. These changes are the result of irritants, toxins, carcinogens etc. present in tobacco leaves and also because of drying effects of the mucosa the high temperatures, intraoral pH changes, immune changes and viral response [15].

Tobacco use is also linked to other types of oral health problems, ranging from serious (increased risk of oral cancer) to social (bad breath) including:

- Gingivitis, periodontal disease, gingival diseases like ANUG, gingival recession
- Sticky tar deposits on teeth, brown staining on teeth
- "Smoker's palate"(a red inflammation on the roof of the mouth)
- Delayed wound healing
- Tooth abrasion, attrition
- Candidiasis
- Altered taste
- Bad breath
- Black hairy tongue
- Precancerous changes in soft tissue [16]

Microbial effect: Tobacco use alters the quality of the oral microflora. The oxygen tension in the periodontal pocket is lower in smokers which favours the growth of anaerobic species. Studies carried out for changes in microbial flora among smokers and non-smokers do not substantiate this [17].

ANUG is strongly correlated with tobacco use. 90% of ANUG patients are smokers. The exact cause of ANUG is, of course, unknown, but it occurs most frequently in teenagers and young adults and may result from defective neutrophil function allowing bacterial and possibly viral (cytomegalovirus) invasion of gingival tissues. The vasoconstrictive action of nicotine and other tobacco components is thought to contribute strongly to the painful tissue necrosis and ulceration seen in this disease, but emotional stress and poor oral hygiene also play important roles [15].

Methodology

Source of data

Patients reporting to the OPD at Department of Oral Pathology and Oral Medicine at Dr Z A Dental College, AMU Aligarh were included in the study. Informed consent from the patient was obtained prior to the study.

Method of collection of data

The study included 150 patients who were subdivided as:

1. **Group I:** 50 subjects with a habit of chewing tobacco more than 5 times a day for a period of 5 years or more, without any history of smoking.
2. **Group II:** 50 subjects with a habit of bidi smoking at least 20 times a day for a period of 5 years or more, without any history of chewing tobacco.
3. **Group III:** 50 normal individuals with negative history of tobacco use (Controls).

Inclusion Criteria

1. Subjects/individuals in the age of 20 - 40 years were included.

Exclusion Criteria

1. Subjects with oral lesions like Leukoplakia, OSMF, Oral Lichen Planus etc.
2. Subjects having any systemic diseases like Diabetes Mellitus, Hypertension, etc.
3. Subjects taking antibiotic therapy.
4. Subjects consuming alcohol.

The estimation of salivary pH and amylase was carried out in the Department of chemistry and Department of Zoology Aligarh Muslim University Aligarh.

Collection of samples

Saliva samples were obtained between 9:00 am to 12:00 pm to avoid diurnal variation. Subjects were advised to avoid eat/ drink or smoke an hour prior to saliva collection. Subject were required to accumulate saliva in the mouth for about 2 minutes followed by spitting the accumulated saliva in a sterile plastic container. This procedure should be repeated form6 minutes for every subject and the unstimulated saliva should be refrigerated at 4^{o}C and sent to processing within 24 hours.

Procedure for evaluation of biochemical constituents of saliva

Salivary amylase was estimated by commercially available Chema Diagnostic's kit.

Estimation of salivary amylase

Kit: Chema diagnostics.

Principle

The enzyme α- amylase (EC3.2.1.1,1,4α-D-glucose glucanohydrolase) hydrolyzes the 2-chloro-4-nitrophenyl- α-D-maltotrioside (CNPG3) to release 2-chloro-4-nItrophenol and form 2- chloro-4-nitrophenyl-α-D-maltoside (CNPG2), maltotriose (G3) and glucose (G). The rate of formation of the 2-chloro-4-notrophenolcan is detected spectrophotometrically at 405 nm to give a measurement of α-amylase activity in the sample.

Test procedure

Wavelength: 405 nm
Light path: 1 cm
Temperature: 37^{0}C
Dispense in cuvette working reagent: 1 ml
Preincubate at 37^{0}C for 5 minutes
Add saliva: 25 µl

Mix, execute a first reading of absorbance after 1 minute, incubating at 37^{0}C. Perform other 3 readings at 60 seconds interval. Calculate the ΔA/min.

Results calculation

Calculated in units per litre, multiplying the ΔA/min by the factor as is indicated.

Calculation in U/l: ΔA/min X 3178.

Statistical analysis

Statistical analysis was done using SPSS software version 13.1. For various parameters mean and standard deviation were calculated. The comparisons of each parameter were statistically compared using One Way Analysis of Variance (ANOVA) and pair wise comparison by post hoc TUCKY test.

Results and Observation

The present study included 150 subjects, divided in three groups as bidi smokers, khaini chewers and normal controls. Data analysis using SPS software version 13.1. For various parameters in three groups, mean and standard deviation were calculated.

The comparisons of each parameter for three groups were statistically compared using one way analysis of variance (ANOVA). Further pair wise comparison is made using the *post hoc* TUKEY test.

Parameters	Bidi smokers		Khaini chewers		Normal control	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Salivary amylase	1163.93	81.87	988.37	91.31	1508.57	160.12

Table 2: Comparison of various parameters in each group.

Parameter		Sum of Squares	Degree of freedom	Mean Square	F	Sig.
Salivary Amylase	Between Groups	5017237.56	2	2508618.78	184.994352	7.19394E-41
	Within Groups	1993395.78	147	13560.51551		
	Total	7010633.34	149			

Table 3: ANOVA for salivary amylase.

Parameter	Comparison between groups	Mean Difference(I-J)	P value	Inference
Salivary Amylase	Smokers and chewers	79.55	0.00446	S
	Smokers and controls	-374.65	5.1E-09	S
	Chewers and controls	-430.56	5.1E-09	S

Table 4: Comparison of the salivary amylase in different subgroups (TUKEY analysis).

Table shows comparative analysis of salivary amylase among different subgroups using Tukey analysis. Salivary amylase was significantly reduced in bidi smokers and khaini chewers in comparison to controls with 'p' value < 0.0001, and also there was significant reduction in salivary amylase in khaini chewers as compared to bidi smokers with 'p' value of .0042.

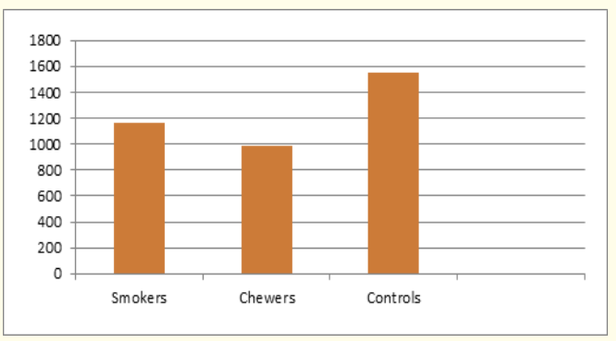

Figure 1: Comparison of salivary amylase in different subgroups.

Discussion

Saliva has an important function in oral health maintenance and regulating the integrity of oral mucosa [18]. Salivary diagnosis is an easily obtainable, non-invasive increasingly important field in dentistry. It is evident that many systemic diseases and local conditions affect salivary gland function and salivary composition. Any change in the production or composition of saliva can increase the mucosal permeability especially with the use of tobacco and predispose it to oral cancer. Decreased salivary output can have deleterious effects on oral and systemic health.

This study is aimed at evaluating the changes in the biochemical composition of saliva in tobacco chewers and tobacco smokers among Aligarh population. The present study comprised of 150 subjects, among them 50 subjects had a habit of bidi smoking, 50 subjects had a habit of khaini chewing and 50 healthy subjects (no smoking or chewing habit) or the control group. Patients in the study are all Male subjects (age group of 20 - 40) considering the fact that habit of tobacco is more prevalent in young age and in male gender specifically [19].

Saliva samples were obtained between 9:00 am to 12:00 pm to avoid diurnal variation. Subjects were advised to avoid eat/drink or smoke an hour prior to saliva collection. Subject were required to accumulate saliva in the mouth for about 2 minutes followed by spitting the accumulated saliva in a sterile plastic container. This procedure should be repeated 6 minutes for every subject and the unstimulated saliva should be refrigerated at 4°C and sent to processing within 24 hours.

In the study salivary amylase was significantly reduced in both tobacco chewers and smokers. This could be because of the injury to ductal secretory unit as a result of tobacco related toxic products. The reduction in salivary amylase can be attributed to the effect of increase salivary flow [1] and it has also been found that long term exposure to tobacco smoke cause reduction in zymogen granules and vacuolization of salivary acinar cells [18]. There was also a significant decrease in salivary amylase in tobacco chewers as compared to controls which could be attributed to increase salivary flow and thereby causing dilution of the tobacco products. The results found so far were in agreement with the results of other studies done till date [19].

Conclusion

Thus the present study proved significant alterations in the biochemical constituents of saliva in tobacco smokers and chewers. The most possible explanation could be because of the changes in the oral epithelium which increases mucosal permeability leading to more intake of irritants and carcinogens. The other correlation could be due to injury to the secretory unit caused by tobacco related toxic products. However, further studies with an increased sample size should be carried out to determine the precise role of saliva in maintaining the integrity of oral mucosa in health and diseased state.

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