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Original Article

Dentistry Section

Influence of Tobacco Chewing and Smoking on the Salivary Total Antioxidant Power-A Clinical Comparative Study

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ABSTRACT

Introduction: Both smoking and tobacco chewing are associated with increased risk of oral cancer due to the imbalance in the free radicals and antioxidants. Saliva is the first biological medium encountered during tobacco chewing and smoking. Evaluation of total antioxidant power in saliva helps in understanding the risk of oral cancer.

Aim: To assess the effect of tobacco chewing and smoking on salivary flow rate, pH and salivary total antioxidant power.

Materials and Methods: A comparative study was done on male subjects (35-50 years old) accompanying the patients attending Narayana Medical and Dental Hospitals, Andhra Pradesh, India. Unstimulated saliva samples were collected from 45 subjects, categorised as chewers (15), smokers (15), and healthy controls (15) using spitting method. Salivary flow rate, pH and total antioxidant power were determined. The salivary total antioxidant power was measured by using Ferric Reducing Antioxidant Power (FRAP) assay. Analysis of Variance (ANOVA) was used for comparison of three groups with respect to salivary total antioxidant power, flow rate, pH. Tukeys post-hoc analysis was used for pairwise comparison of study groups with respect to salivary total antioxidant power, flow rate and pH.

Results: Salivary total antioxidant power was lowest in tobacco chewers (407±48), compared to smokers (573±60) and controls (800±67). Salivary flow rate was lowest in tobacco chewers (1.43±0.70), compared to smokers (2.31±0.65) and controls (3.09±0.48). Salivary pH was lowest in tobacco chewers (6.34±0.25), compared to smokers (6.73±0.17) and controls (7.05±0.20). The mean difference between the three groups was statistically significant (<0.001).

Conclusion: The evidence of the decreased salivary antioxidants in the tobacco chewers and smokers emphasises the role of smoking and tobacco chewing in the pathogenesis of oral cancers.

Keywords: Mouth neoplasms, Saliva, Tobacco products

INTRODUCTION

Cancer is one of the most common causes of mortality and morbidity nowadays. Oral cancer accounts for approximately 30-40% of all cancers in India. The strong association between oral cancers with tobacco use is well established [1].

Tobacco consumption has a direct correlation with Deoxyribonucleic Acid (DNA) damage. When a cell with DNA damage divides, metabolism and duplication of cells become deranged and mutations can arise, which is an important factor in carcinogenesis [2]. Reactive Oxygen Species (ROS), free radicals and reactive nitrogen species liberated during cigarette smoking and tobacco chewing initially will cause dysplastic lesions which then transform into carcinoma lesions [3]. Free radical formation is naturally controlled by antioxidants. Antioxidants are capable of deactivating or stabilising free radicals before they injure cells. Recently, it has been demonstrated that the imbalances in free radical levels and ROS with antioxidants may play a key role in the onset and development of several inflammatory oral pathologies [4]. Antioxidants are present in all body fluids including saliva. Saliva is the first line of defence against oxidative stress and it has defensive effects against microorganisms, toxins and oxidants [5].

The human body has non-enzymatic and enzymatic antioxidant defence mechanisms to remove harmful ROS. The non-enzymatic antioxidants include reduced glutathione, albumin, vitamins A, C, and E, uric acid, bilirubin, lactoferrin, ceruloplasmin, transferrin, and haptoglobin. The enzymatic antioxidants include glutathione peroxidase, superoxide dismutase and catalase [6]. A delicate balance exists between the pro-oxidant mechanisms of tissue destruction and antioxidant defence repair systems; if the balance is shifted in favour of ROS activity, significant tissue damage occurs [7].

In present study, we assessed total antioxidant power of saliva by using FRAP analysis [8]. As it is suggested that free radicals and antioxidant system appear to act in conjunction rather than alone and measurement of any individual antioxidant may be less representative of whole antioxidant status. Moreover, the number of different antioxidants makes it difficult and also expensive to measure each of them separately [9].

It has been long recognised that saliva serves as a mirror of the body's health as it contains hormones, proteins, and other molecules which are often measured in standard blood tests to detect health and disease. However, unlike blood, collection of saliva is easy and less painful to the patient and is less infectious for the healthcare provider during handling [10]. Healthy individuals produce about a litre and a quarter of saliva per day. Nearly all analytes that are in blood are also present in saliva [11].

Evaluation of total antioxidant power in saliva of tobacco chewers, smokers and healthy controls can pave way in understanding the risk of oral cancer due to the both smokable and chewable tobacco consumption. As for the literature available, very little has been discussed about influence of tobacco chewing and smoking on salivary antioxidants. Therefore, the aim of the present study was to assess the effect of tobacco chewing and smoking on salivary flow rate, pH and salivary total antioxidant power.

MATERIALS AND METHODS

A clinical comparative study was conducted at Narayana Dental College and Hospital for a period of 1 month (September to October 2016) to assess the effect of tobacco chewing and smoking on the salivary total antioxidant power among the male subjects (35-50 years old), who satisfied the inclusion criteria and who gave a written consent. Study subjects were selected from the people

visiting Narayana Medical and Dental Hospitals conveniently, who were not patients (people accompanying the patients). The ethical clearance for the study was obtained from the Institutional Review Board of the Narayana Dental College and Hospital, Nellore.

To estimate the sample size, a power analysis was performed based on the data obtained from a previous study conducted by Jenifer HD et al., with standard deviations of 8.11, least detectable difference of 9.74 [7]. The sample size arrived was 11.64, which was rounded off to 15. Therefore, sample size for each group was 15. As we had three groups, a total of 45 subjects were included in this study.

Inclusion Criteria

Tobacco chewers: Subjects with a history of daily consumption of chewable tobacco for at least 10 years, with no habit of smoking.

Smokers: Subjects with a habit of smoking for at least 10 years and with no habit of tobacco chewing.

Controls: Subjects with no habit of tobacco chewing and smoking in their life time.

Exclusion Criteria

Subjects with both smoking and chewing habit, subjects with oral mucosal lesions and systemic diseases, subjects with a history of chronic alcohol consumption and dental caries and subjects with clinical attachment loss of less than 4 mm were excluded from the study.

Study Procedure

Saliva collection: Unstimulated saliva sample was collected by spitting method [12]. Saliva was collected while the subject was sitting upright with the head slightly tilted forward and the eyes open. The patient was advised to refrain from intake of any food or beverage (water exempted) one hour before the test session. Smoking, chewing gum and intake of coffee were prohibited during this hour. The subject was advised to rinse his mouth several times with deionized (distilled) water and then to relax for five minutes. Saliva was allowed to accumulate in the mouth and subject spitted into the graduated test tube every 60 seconds for five minutes. So that salivary flow rate can be measured.

Salivary pH assessment: The pH of the saliva was assessed using digital pH meter (Ri, model 152-R). In between each reading the electrode was cleaned with a stream of distilled water and placed in a standard solution of pH 7.0. This ensured stable readings and a constant check on drift.

Storage of saliva: Then saliva samples were immediately centrifuged at 800 g for 10 minutes at 4°C to remove cell debris. The resulting supernatants were immediately deep-frozen at -80°C and stored for later analysis.

Total antioxidant power of saliva: Total antioxidant power of saliva was measured by FRAP assay. FRAP was used to determine the antioxidant potential in a given sample. This assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. It gives putative index of antioxidant, or reducing potential of biological fluids within the technological reach of laboratory and researcher interested in oxidative stress and its effects. FRAP utilises the reducing potential of the antioxidant to react with a Ferric Tripyridyltriazine (TPTZ) complex. This produces a coloured ferrous tripyridyltriazine form. The change in absorbance at 593 nm can then be compared with a standard to determine the antioxidant potential in a given sample [8].

STATISTICAL ANALYSIS

The collected data were analysed using Statistical Package for the Social Sciences (SPSS) version 20.0. Analysis of Variance (ANOVA) was used for comparison of three groups with respect to salivary total antioxidant power, flow rate and pH. Tukeys post-hoc analysis was used for pairwise comparison of study groups with respect to salivary total antioxidant power, flow rate, pH. A p-value less than 0.05 was considered as statistically significant.

RESULTS

A total of 45 subjects participated in the study with 15 each in smoker group, tobacco chewer group and control group with a mean age of 44.23±4.001. All the participants were males and there was no significant difference in the mean age between the three study subjects so they were comparable [Table/Fig-1]. The mean duration of smoking was 16.73±3.411 years and mean duration of tobacco chewing was 14.53±4.138 years among the smokers and chewers respectively.

Salivary total antioxidant power was lowest in tobacco chewers (407±48), compared to smokers (573±60). Controls (800±67) had the highest total antioxidant power. This difference was statistically significant. Post-hoc assessment for one to one comparison confirmed a statistically significant difference between smokers vs. chewers, smokers vs. controls and chewers vs. controls in terms of total antioxidant power, indicating that tobacco chewers had significant lower total antioxidant power compared to other groups [Table/Fig-2].

Salivary flow rate was lowest in tobacco chewers (1.43±0.70) compared to smokers (2.31±0.65). Controls had (3.09±0.48) the highest salivary flow rate. This difference was statistically significant. Post-hoc assessment for one to one comparison confirmed a statistically significant difference between smokers vs. chewers, smokers vs. controls and chewers vs. controls in terms of salivary flow rate [Table/Fig-3]. Salivary pH was lowest in tobacco chewers (6.34±0.25) compared to smokers (6.73±0.17). Controls had the

Group	N	Mean age (years)	Std. Deviation	ANOVA	
				F	p-value
Tobacco smokers	15	44.07	3.85	0.15	0.86
Tobacco Chewers	15	44.40	4.44		
Controls	15	43.60	3.72		

[Table/Fig-1]: Age distribution of study groups.
p>0.05=Non significant

Group	TAP Mean±SD (µmol/L)	ANOVA	Post-hoc analysis					
		F (p-value)	(I) Group	(J) Group	Mean Difference (I-J) (SE)	p-value	95% CI	
							Lower bound	Upper bound
Tobacco smoker	573±60	167.07 (<0.001*)	Tobacco smoker	Tobacco chewer	166 (0.22)	<0.001*	114	219
				Controls	-227 (0.22)	<0.001*	-280	-175
Tobacco chewer	407±48		Tobacco chewer	Controls	-393 (0.22)	<0.001*	-446	-341
Controls	800±67							

[Table/Fig-2]: Comparison of study groups with respect to salivary total antioxidant power.
*p<0.05 Statistically significant; TAP: Total antioxidant power
SD: Standard deviation; SE: Standard error

highest pH (7.05±0.20). The difference was statistically significant. Post-hoc assessment for one to one comparison confirmed a statistically significant difference between smokers vs. chewers, smokers vs. controls and chewers vs. controls in terms of pH [Table/Fig-4]. This Indicates a significant lower salivary flow rate and pH among tobacco chewers compared to others.

The most important and interesting finding of the present study was that salivary total antioxidant power of smokers and tobacco chewers was decreased compared to healthy controls. The findings of the current study were similar to the findings of the other studies. Study conducted by Falsafi P et al., showed that the levels of antioxidants in smokers were lower than non-smokers [18]. Study conducted

Group	Salivary flow rate Mean±SD (mL/5 minute)	ANOVA	Post-hoc analysis					
		F (p-value)	(I) Group	(J) Group	Mean Difference (I-J) SE	p-value	95% CI	
							Lower bound	Upper bound
Tobacco smoker	2.31±0.65	26.84 (<0.001*)	Tobacco smoker	Tobacco Chewer	0.87 (0.23)	0.001*	0.33	1.42
				Controls	-0.78 (0.23)	0.004*	-1.33	-0.23
Tobacco chewer	1.43±0.70		Tobacco chewer	Controls	-1.65 (0.23)	<0.001*	-2.20	-1.11
Controls	3.09±0.48							

[Table/Fig-3]: Comparison of study groups with respect to salivary flow rate.
*p<0.05 Statistically significant,
SD: Standard deviation,; SE: Standard error

Group	Salivary pH Mean±SD	ANOVA	Post-hoc analysis					
		F (p-value)	(I) Group	(J) Group	Mean Difference (I-J) (SE)	p-value	95% CI	
							Lower bound	Upper bound
Tobacco smoker	6.73±0.17	26.84 (<0.001*)	Tobacco smoker	Tobacco chewer	0.39 (0.08)	<0.001*	0.21	0.58
				Controls	-0.32 (0.08)	<0.001*	-0.51	-0.14
Tobacco chewer	6.34±0.25		Tobacco Chewer	Controls	-0.71 (0.08)	<0.001*	-0.90	-0.53
Controls	7.05±0.20							

[Table/Fig-4]: Comparison of study groups with respect to salivary pH.
*p<0.05 Statistically significant; SD: Standard deviation; SE: Standard error

DISCUSSION

Initially clinical examinations and health questionnaires were used to identify patients at risk of developing diseases. Haematologic, serologic and imaging diagnostic methods were used to assess these patients further. In recent years, saliva-based diagnostic tests have been increased because of their non-invasive nature. Technologies are available that use saliva to diagnose, follow and assess the risk and severity of diseases [13]. Given the importance of saliva and its defence system, and due to the limited studies in this field, the present study was aimed to assess the effect of tobacco chewing and smoking on the salivary total antioxidant power.

In the present study, total antioxidant power of the saliva was measured by using FRAP assay. FRAP reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL Ferric TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution. It is a novel method for assessing "antioxidant power." Ferric to ferrous ion reduction at low pH causes a coloured ferrous-tripyridyltriazine complex. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration [8].

Age influences the total antioxidants status of saliva. As elderly people have significantly reduced total salivary antioxidant capacity [14]. We included this particular age group in the current study. Subjects with dental caries were excluded in this study, as the total antioxidant capacity in saliva of children with early childhood caries will be significantly greater than in the group without caries [15]. Subjects with extensive periodontitis and chronic alcoholics were also excluded in this study, as periodontal disease is associated with reduced salivary antioxidant status [16] and the alcohol-dependent subjects showed significantly lower total antioxidant capacity in saliva compared to the controls [17].

by Greabu M et al., showed that total antioxidant capacity of saliva was lower in smokers than non-smokers [19]. The study conducted by Shetty AV et al., showed that smokers with and without caries showed decrease in total antioxidant level compared to the non smokers with and without caries [20]. Patel T et al., observed that oral submucous fibrosis patients consuming tobacco quid showed low levels of antioxidant enzymes than the healthy controls [1].

Free radicals are very unstable and react quickly with other compounds. Antioxidants can neutralize free radicals by donating their electrons [9]. A comprehensive mechanism is suggested for the initiation of oral cancer by cigarette smoking, saliva loses its antioxidant capacity and becomes a potent pro-oxidant in the presence of cigarette smoke. This mechanism is based on the well-known observation that oral cancer mostly occurs in oral epithelial cells exposed to tobacco products [3].

In present study, salivary flow rate and pH were lowest in the tobacco chewers and smokers compared to controls, which was similar to study conducted by Kanwar A et al. They showed that salivary flow rate decreases appreciably among tobacco users especially more among smokeless form. A lower salivary pH was observed in tobacco users than in controls. Decrease in salivary flow rate in smokers and chewers are probably due to the effect of nicotine on the taste nerve apparatus. Decrease in pH may be due to the alteration in electrolytes and ions as they interact with the buffering systems of saliva [21].

Saliva is the first body fluid which encounters the cigarette smoking and tobacco chewing. Antioxidant system of saliva plays a significant role in the anticariogenic and antibacterial effect of saliva. Results of the present study indicated that oxidant-antioxidant balance of saliva is degraded in favour of free radicals. Degradation of this oxidant-antioxidant balance possibly will contribute to worsening of

oral hygiene and oral cancer development in smokers and tobacco chewers.

LIMITATION

Main limitation of present study was that smoking status was recorded by the self-reporting of subjects. However, estimation of nicotine intake by serum cotinine assay would give more reliable information regarding tobacco consumption. As salivary antioxidants in smokers and chewers were significantly decreased, it is recommended to incorporate antioxidants in food supplements, tooth pastes and mouth rinses in order to prevent the harmful effects of tobacco.

CONCLUSION

Tobacco chewers and smokers had a lower salivary total antioxidant power compared to controls. This evidence of the decreased salivary antioxidants in the tobacco chewers and smokers emphasises the role of smoking and tobacco chewing in the pathogenesis of oral potentially malignant disorders and cancers.

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