

In vitro Antioxidant Assessment of Saliva from Non Smokers and Smokers

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Abstract

The assay of saliva is an increasing area of research with implications for basic and clinical purposes. Although this biological fluid is easy to manipulate and collect, careful attention must be directed to limit variation in specimen integrity. Recently, the use of saliva has provided a substantial addition to the diagnostic armamentarium as an investigative tool for disease processes and disorders. In this study the antioxidant activity of saliva from smokers and non smokers of both the sex of various age groups were assessed. The FRAP assay revealed that the reducing power of non smokers were high when compared to that of both the acute and chronic smokers. Other antioxidant assays like CUPRAC and Ce (IV) sulphate assay also supported the FRAP assay. A two way ANOVA for all the *in vitro* antioxidant assays proved to be significant at 5% level ($p < 0.05$) between the smokers and non smokers. Overall, it was observed that a high reduction in the antioxidant power in the smokers may later on, leads to SCC

Keywords: Saliva, antioxidant, Cigarette smoke (CS), Oral squamous cell carcinoma (SCC), oral cancer, FRAP

1. Introduction

Atmospheric oxygen is a diatomic molecule with the formula O_2 , in which the two oxygen atoms are doubly bonded to each other (triplet oxygen). This form of oxygen has two unpaired electrons, making it as a radical. Whereas most radicals are highly reactive molecules, triplet oxygen is fortunately unreactive (Navdeep *et al.*, 2007). Approximately 5% of O_2 involved with normal processes like metabolic respiration, strenuous exercise and biotransformation of xenobiotics (Peter Møller and Steffen Loft, 2006) is responsible for the generation of free radicals or reactive oxygen species (ROS). A free radical is a molecule containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell and Gutteridge, 1999) that includes superoxide ions (O_2^-),

hydroxyl radicals (OH) and H_2O_2 (Mazumder *et al.*, 2006; Zeynep Alpay *et al.*, 2006). These ROS may induce oxidative damage to various macromolecules like polyunsaturated fatty acids in cell membranes (Halliwell and Gutteridge, 1999), carbohydrates, proteins and DNA (Stadtman, 1992), which results in homeostatic imbalance (Bonfont *et al.*, 2000).

Cigarette or tobacco smoke is involved in the pathogenesis of several diseases regarding different body systems, mainly cardiovascular and respiratory in addition to its local toxic effect in the oral cavity. The noxious effects of smoke compounds justify the high incidence of periodontal diseases, caries and neoplastic diseases of oral tissues in smokers (Zappacosta *et al.*, 2002). There are numerous harmful substances found in the tobacco smoke and nicotine is one of the main

substance that may be acquired through active and passive smoking (Ross Cooper, 2006). Moreover, nicotine is one of the most important molecule that is responsible for the compulsive use of tobacco (Maurizio Battino *et al.*, 2007). The two phases of cigarette combustion product can be distinguished as the gas phase and the solid particulate phase (also known as tar phase). Particulate is the material retained on a filter, whereas gas phase smoke passes through the filter. In fact, cigarette smoke (CS) contains over 4,000 different chemicals, out of which 3,000 are in the gas phase and 1,000 in the particulate phase. Four hundred of them have been proven to be carcinogens (Maurizio Battino *et al.*, 2007). Both the particulate and gas-phase smoke are very rich sources of free radicals (Halliwell and Gutteridge, 2007). Oral squamous cell carcinoma (SCC) is the most common malignancy of the head and neck, with a worldwide incidence of over 300,000 new cases annually. One of the major inducers of SCC is exposure to cigarette smoke (CS), responsible for 50-90% of the cases (Maurizio Battino *et al.*, 2007). CS, which contains several carcinogens are known to initiate, promote and metastasis of oral cancer (Nagaraj and Zacharias, 2007). The incidence of SCC in cigarette smokers is four to seven times higher than in non-smokers, when alcohol is also consumed this incidence is even higher. Moreover, compared with non-smokers, the higher cigarette smoke-related risk for SCC is manifested by a reduction in the mean age of development of the disease by 15 years (Nagler and Abraham Z. Reznick, 2004).

Saliva in humans is a mouth fluid possessing several functions involved in oral health and

homeostasis with an active protective role in maintaining oral healthiness. It is a complex secretion whose components exert a well-documented role in health and disease (Johan K.M. Aps and Luc C. Martens, 2005). It also facilitates taste perception, allowing soluble food-derived molecules to reach the gustative papillae and buffer the acid components of food with the bicarbonates (originating from salivary gland carbonic anhydrase). Saliva also has a role in maintaining teeth enamel mineralization with the help of several proteins like statherin, proline rich proteins (PRPs) and mucins (Nurdan Ozmeric, 2004). In addition to its lubricant properties, saliva contains many biochemical systems known to be involved in soft-tissue repair, and many antibacterial components (Bruno Zappacosta *et al.*, 1999) including lysozyme, lactoferrin and salivary peroxidase (Carlsson, 1987). Furthermore, saliva contains various antioxidants, including uric acid, which contributes almost 70% of the total radical-trapping antioxidant capacity (Moore *et al.*, 1994).

The anticarcinogenic capability of saliva was shown to significantly inhibit the initiation and progression of oral cancer in an animal model (Dayan *et al.*, 1981). Saliva is the first biological medium met by external materials taken into the body as part of food, drink, or inhaled volatile ingredients. During evolution, various defense mechanisms developed in the saliva aimed at combating penetrating bacteria, viruses or fungi and protecting against chemical or mechanical attack (Nagler and Abraham Z. Reznick, 2004). Recently, the importance of an additional salivary defense system has become clear (Kohen *et al.*, 1992), i.e., the antioxidant defense system, which appears to lose efficiency with advanced age. Similar to other biological systems, the salivary antioxidant system includes various molecules and enzymes that can be used as biomarkers for diagnosis of various periodontal diseases are summarized in Table 1 (Nurdan Ozmeric, 2004). The main aim of the present study carried out in our laboratory has been focused in evaluating the antioxidant power of the saliva in both smokers and non smokers of various age groups and to correlate the possibility of occurrence of oral cancer among the smokers.

Table 1 Salivary markers for diagnosis of periodontal diseases

Enzymes	Proteins	Immunoglobulins	Others
Elastase	Lactoferrin	IgA	Urate
Amylase	Fibronectin	IgG	Ascorbic acid
Arginase	Albumin	IgM	Platelet activating factor
Lysozyme	Cystatins C, S and A		Cortisol
Myeloperoxidase	Histatins		Nitrite
Chitinase	Keratin		Bilirubin
Oral peroxidase	EGF		Hyaluronic acid
Myeloperoxidase	VGF		Chondroitin sulphate

2. Materials and Methods

2.1 Preparation of salivary sample

Mixed saliva (about 5 ml) was collected 2-3 hrs after breakfast from both the smokers and non smokers. The sample was centrifuged for about 15 minutes at 16,000 rpm for 5 minutes to remove the cellular components. The supernatant obtained was used for the *in vitro* antioxidant studies.

2.2 Ferric ion reducing/ antioxidant power assay (FRAP)

A method developed by Oyaizu (1988) was adopted for the determination of reducing power. About 0.02ml to 0.1ml of the saliva was made up with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide was added to the reaction mixture. The mixture was incubated in a 50°C water bath for 20 minutes, then rapidly cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 3000 g for 10 minutes. Pipetted out 2.5 ml of the supernatant, 2.5 ml of distilled water and 0.5 ml of 0.1 % ferric chloride and mixed well. Allowed to stand for 10 minutes and the Prussian blue colored complex formed was spectrophotometrically measured at 700 nm. This was used to evaluate the reducing power of the salivary sample.

2.3 Modified Cupric ion reducing/ antioxidant power assay (CUPRAC)

The method proposed by Resat apak *et al.*, (2006) was espoused. About 0.02ml to 0.1ml of the saliva was made up with 2.5 ml distilled water. To this added 1ml of cupric chloride, 1ml of ammonium acetate, 1 ml of neocuproine and finally, 1ml of absolute

ethanol. The mixture was incubated at room temperature for 30 minutes and the yellow colored product formed was measured. The increase in the absorbance at 450 nm was used to measure the cupric ion reducing power of the salivary sample.

2.4 Cerium (IV) sulphate assay

A method developed by Resat Apak *et al.*, (2007) was adopted for the determination of reducing power. About 0.02 ml to 0.1ml of the saliva was made up with 1.0 ml distilled water. To this added 1.0 ml of 0.002 M Ce (IV) sulphate solution. After shaking for a few minutes, the solution was let to stand for 30 minutes at room temperature. The absorbance of the reaction mixture was measured at 320 nm against a blank composed of distilled water. The decrease in the absorbance at 320 nm was used to measure the unreacted Ce (IV) ion in the salivary sample, which indicates an increase in antioxidant power of the sample.

3. Results and Discussion

It has been suggested that free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the inhaled cigarette smoke induce a gradually evolving process, initially expressed by dysplastic lesions of the mucosa, which are transformed into *in situ* carcinoma lesions and eventually result in full blown infiltrating and metastasizing SCC (Maurizio Battino *et al.*, 2007). FRAP assay involves neither an oxidant nor an oxidizable substrate but it measures the ability to reduce the ferric ions present in the assay mixture to ferrous ions. Even if the iron ions are given exogenously in the assay, in order to magnify the

phenomenon, this test gives valuable information about the reductants present in biological fluids (Vassalle *et al.*, 2004). Furthermore, the cigarette smoke contains certain metal ions (such as iron) that can catalyze the production of hydroxyl radicals from hydrogen peroxide via the Fenton reaction.



An increased absorbance as a function of increased volume was noticed and it indicated saliva's high reducing potentiality. The antioxidant capacity of the saliva of CS (acute and chronic) was drastically decreased when compared to that of non smokers (Fig.1). Even though there is an elevation of antioxidant power in the acute smokers as a function of sudden cigarette smoking, a sudden extreme decrease is noticed due to a continual smoking. Statistical analysis at 5% level was not proved to be significant ($p < 0.05$) between the non smokers salivary sample. But, the standard error was found to have a trifling deviation (0.0058) proving a high reducing power. A high correlation between the volume and the absorbance was observed according to Karl Pearson Correlation of analysis (0.997). It was also observed that the smoker's samples (Both acute and chronic) possess a drastic decrease in the antioxidant activity and a high correlation (Karl Pearson Correlation of analysis, 0.997) between the antioxidant capacity and smoking was observed. Student's t- Test analysis revealed a significant difference at 5% level ($p < 0.05$) between the smokers of both type and the non smokers. A two way ANOVA analysis performed by a software Originpro, proved a significant difference at 5% level ($p < 0.05$) among the non smokers, acute and chronic smokers (Table 2). The production of hydroxyl radical occurs in vitro occurs even due to the presence of Cu^{2+} (Marian Volko *et al.*, 2006). In this aspect recently, Res at Apak *et al.*, (2006) proposed a method to evaluate the cupric ion reducing power present in the samples. An increased absorbance as a function of increased volume was noticed and indicated saliva's high reducing potentiality. The standard error was

Table 2 - Two way ANOVA (Reducing power Assay)

	DF	Sum of Squares	Mean Square	F value	P value
Factor A	1	1.40002E-5	1.40002E-5	0.02191	0.88408
Factor B	1	0.0023	0.0023	6.59419	0.07511
Model	2	0.0023	0.00116	1.80805	0.19412
Error	17	0.01086	6.9071E-4	-	-
Corrected Total	19	0.9318	-	-	-

Factor A = Similar volume of saliva between the non smokers, acute and chronic smokers
 Factor B = Different volume of saliva between the non smokers, acute and chronic smokers ($p < 0.05$)

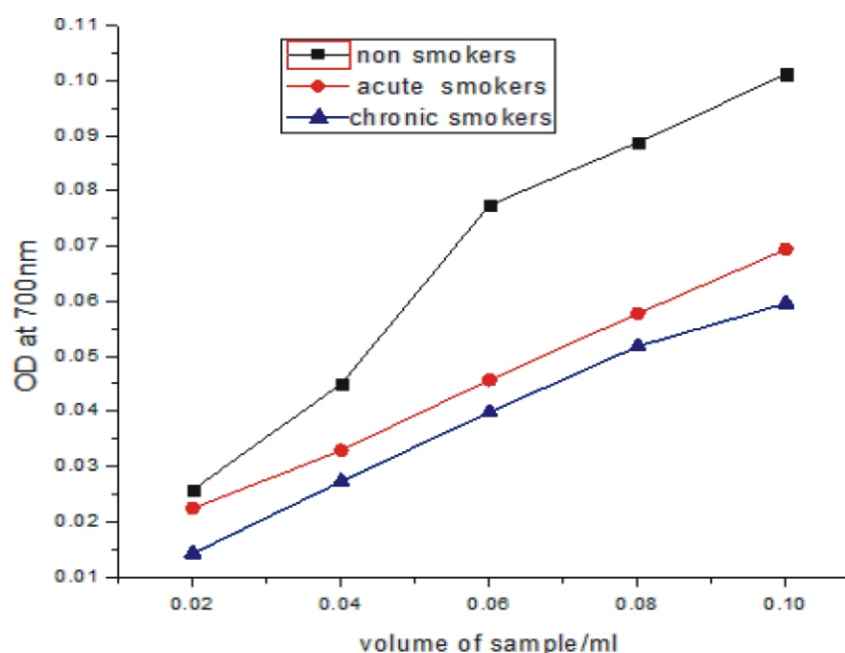


Figure -1 Effect of antioxidant activities of non-smokers, acute and chronic smokers

Table 3 - Two way ANOVA (Modified CUPRAC Assay)

	DF	Sum of Squares	Mean Square	F value	P value
Factor A	1	7.74469E-4	7.74469E-4	1.17021	0.29447
Factor B	1	0.01222	0.01222	18.46776	4.8768E-4
Model	2	0.013	0.0065	9.81898	0.00146
Error	17	0.01125	6.61822E-4	-	-
Corrected Total	19	0.02425	-	-	-

Factor A = Similar volume of saliva between the non smokers, acute and chronic smokers
 Factor B = Different volume of saliva between the non smokers, acute and chronic smokers ($p < 0.05$)

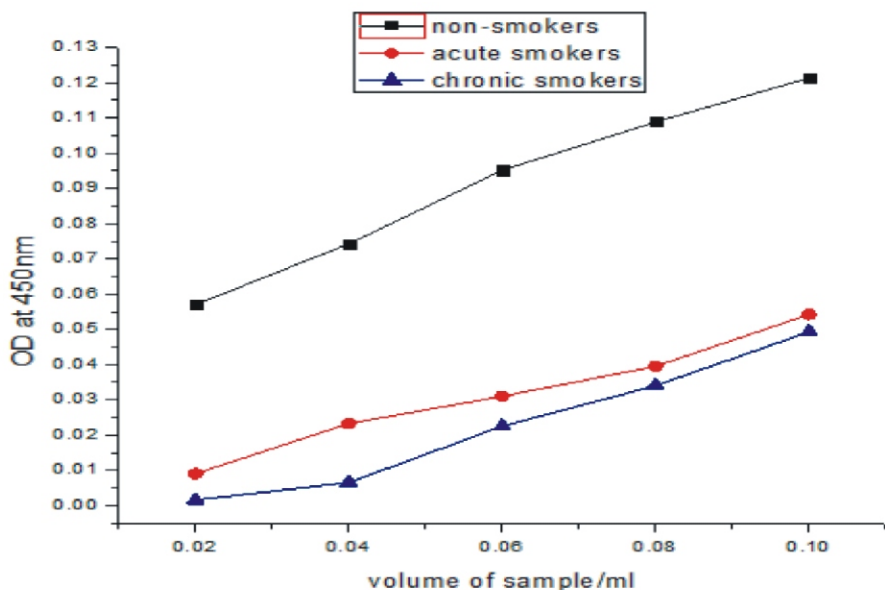


Figure - 2 Effect of antioxidant activities of non-smokers, acute and chronic smokers

Table 4 - Two way ANOVA (Cerium (IV) sulphate Assay)

	DF	Sum of Squares	Mean Square	F value	P value
Factor A	1	0.03727	0.03727	1.16998	0.29451
Factor B	1	0.22542	0.22542	7.07582	0.0164
Model	2	0.26269	0.13134	4.1229	0.03469
Error	17	0.54157	0.03186	-	-
Corrected Total	19	0.80426	-	-	-

Factor A = Similar volume of saliva between the non smokers, acute and chronic smokers

Factor B = Different volume of saliva between the non smokers, acute and chronic smokers ($p < 0.05$)

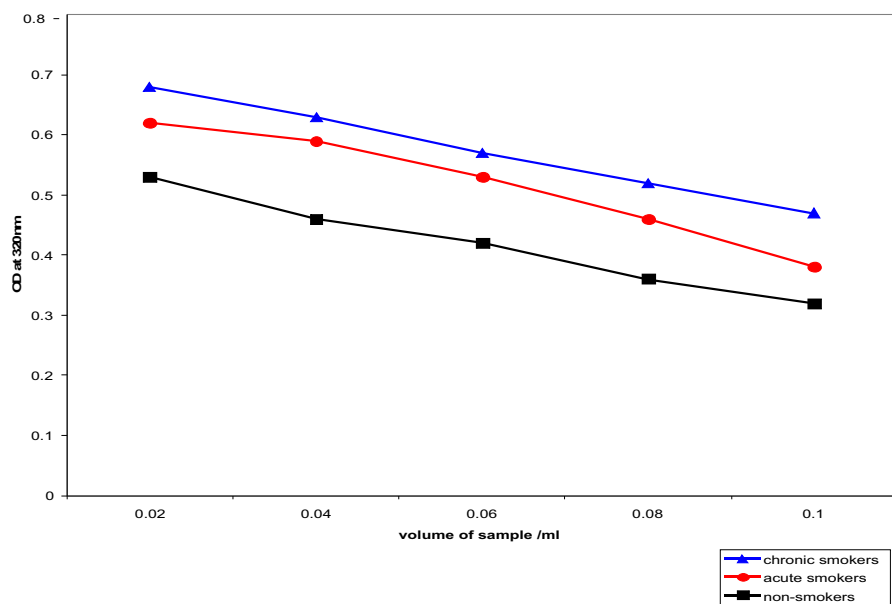


Figure - 3 Effect of antioxidant activities of non-smokers, acute and chronic smokers

found to have a insignificant deviation (0.079) proving a high reducing power. A high correlation between the volume and the absorbance was observed according to Karl Pearson Correlation of analysis (0.998). It was also observed that the smoker's samples possess a drastic decrease in the antioxidant activity and a high correlation (Karl -Pearson Correlation of analysis, 0.998) between the antioxidant capacity and smoking was observed (Fig.2). Student's t- Test analysis revealed a significant difference at 5% level ($p < 0.05$) between the smokers of both type and the non smokers. A two way ANOVA analysis performed by a software Originpro, proved a significant difference at 5% level ($p < 0.05$) among the non smokers, acute and chronic smokers (Table 3).

The Ce (IV) reducing capacity of the sample is measured under carefully adjusted conditions of oxidant concentration and pH such that only antioxidants and not other organic compounds (such as benzoic acid, acetyl salicylic acid, and simple sugars, which do not act as reducing agents) would be oxidized. The advantage of the method is its simplicity, and applicability to conventional laboratories (without high-level instrumentation), added to the possibility of measuring the fluorescence of the Ce(III) produced as a result of the redox reaction concerned, besides absorptimetric measurement of the remaining Ce(IV) (Resat apak et al., 2007). A decreased absorbance as a function of increased volume was noticed and indicated saliva's high reducing potentiality. A high correlation between the volume and the absorbance was observed according to Karl Pearson Correlation of analysis (0.998). It was also observed that the smoker's samples possess a drastic decrease in the antioxidant activity and a high correlation (Karl Pearson Correlation of analysis, 0.998) between the antioxidant capacity and smoking was observed (Fig.3). Student's t- Test analysis revealed a significant difference at 5% level ($p < 0.05$) between the smokers of both type and the non smokers. A two way ANOVA analysis performed by a software Originpro, proved a significant difference at 5% level ($p < 0.05$) among the non smokers, acute and chronic smokers (Table 4).

4. Conclusion

Recently there has been increasing interest in diagnosis based on saliva analyses, because saliva has a simple and noninvasive collection method. Oral fluid sampling is safe for the operator and the patient, and has easy and low-cost storage. In conclusion, it was proved that saliva possess a significant antioxidant activity in the non smokers compared to that of smokers of both types, which has been revealed by all the in vitro antioxidant assays. The two way ANOVA also proved a significant difference between the non smokers, acute and chronic smokers. The studies also revealed that a decreased antioxidant power, in future may progressed to SCC and other related cancers.

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