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Alternations of Antioxidant Activity in Saliva in Smokers

S. F. Nosratabadi[1], R. Sariri[2*], P. Yaghmaei[1], M. Taheri[2], A. Ghadimi[3] and H. Ghafoori[2]

[1] Department of Biochemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Department of Microbiology, Lahijen Branch, Islamic Azad University, Lahijan, Iran
[3] Department of Biochemistry, Kordestan Science and Research Branch, Islamic Azad University, Sanandaj, Iran

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ABSTRACT

Cigarette smoke is injurious to both oral cavity and internal body environment. Saliva is the first body fluid to encounter the dangerous smoke. The aim of our present study was, therefore, to evaluate the influence of smoking on the antioxidant activity of saliva of healthy smokers. Antioxidant capacity of saliva was determined using different methods in the centrifuged saliva from 25 male smokers and 25 non-smokers in a similar age range. Chemical methods and high performance liquid chromatography (HPLC) revealed a significant decrease in total antioxidant capacity and values of non-enzymatic antioxidants in saliva of smokers. Based on the results obtained in the present study, we concluded that smoking could significantly affect the anitoxidant behavior of normal human saliva. Thus, smoking may induce several oral diseases as well as respiratory and cardiovascular disorders and could cause different cancers.

Keywords: Salivary antioxidants; HPLC; Cigarette smoke; DPPH, FRAP

INTRODUCTION

Smoking cigarette introduces many toxic chemicals in the oral cavity causing serious health problems. The toxic compounds reflect their effect as periodontal diseases, caries, and neoplastic diseases of oral tissues in smokers. Some of the important human diseases including cardiovascular and respiratory disorders are also caused or worsen by tobacco smoke [1]. This dangerous smoke contains about 4000 different chemicals, 1/10th of which are known carcinogens. Tobacco smoke also contains potent oxidants such as oxygen free radicals and volatile aldehydes [1, 2]. The oxidizing agents can seriously damage biomolecules such as proteins and enzymes leading to various physiological problems. Saliva is secreted by three pair of major salivary glands as well as by hundreds of minor glands located below the mucosal surfaces of the mouth [3, 4]. Salvia contains a complex mixture of substances, similar to other body fluids [5]. Biochemistry of saliva is a combination of locally produced proteins and enzymes together with some other biochemicals such as antioxidants, electrolytes, epithelial and immune cells, microorganisms, bronchial products. Besides, other external substances with various concentrations are also found in salivary fluid [6]. The use of saliva as source of important biomarkers has recently attracted attention of

* Corresponding author: sariri@guilan.ac.ir

some researchers [7]. It has been shown that biochemical composition can also reflect the causes of some systemic disease [8]. Human saliva can reflect the relationship between oral hygiene and some chronic systemic diseases [9] exercise intensity [10]. Saliva protects oral cavity against dangerous agents such as microorganisms, toxins and various oxidants [11]. However the antioxidant capacity and reducing power of saliva may be reduced due to various factors [12]. It has been shown that *in vitro* exposure to cigarette smoke could significantly decrease biological activity of some enzymes, both in plasma and in saliva [13, 14].

MATERIALS AND METHODS

Chemicals and equipments

2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH). 2,4,6-Tripyridyl-s-triazine (TPTZ), sodium acetate, ferric chloride, gallic acid, Folin-Ciocalteu's phenol reagent and ferrous sulphate were purchased from Sigma representative in Iran. Uric acid and ascorbic acid as well as all solvents were of reagent grade and obtained from Merck representative in Iran. High performance liquid chromatography instrument with diode array detector (HPLC-DAD) and UV-visible spectrophotometer (Ultrospec 3000, Pharmacia Biotech™, Sweden) were also used throughout the research.

Saliva collection, flow rate and storage

25 smokers (15-20 similar cigarettes/day) and 25 non-smokers volunteered to enter into our study. The subjects were male university students 22-25 years old with healthy teeth and gums and did not suffer from any internal or genetic disease. Care was taken that all subjects had similar weight, eating habits and life styles. They signed consent and filled a form about their health background and various aspects of life and history of smoking. They were asked to keep fast for 8 hours before donating their saliva. After rinsing their mouth with distilled water, they collected un-stimulated saliva samples (3 ml) in clean, dry in sterile pre-weighted tubes [10]. The time required to collect 3 ml saliva samples was different for each individual depending on their flow rate (2.0-5.0 minutes). The time in minutes for collecting one ml of saliva was taken as flow rate. The collected samples were immediately centrifuged at 800 × g for 10 min at 4°C to remove squamous cells and cell debris. The resulting supernatant was stored at -18°C until their examination for antioxidants activity.

DPPH radical scavenging assay

In this chemical assay, an antioxidant reduces the stable $DPPH^{\cdot}$ by donating a hydrogen to it. In the present piece of research, a modification of the method described in [15] was used. The radical scavenging activity of saliva samples against stable $DPPH^{\circ}$ was determined spectrophotometrically. The color of $DPPH^{\cdot}$ changed from deep-violet to light-yellow due to its reduction. The color change was measured at 517 nm using a UV/visible light spectrophotometer (Ultrospec 3000 from Pharmacia Biotech). Briefly, 1500 µl of freshly prepared $DPPH^{\circ}$ solution in methanol (6×10^{-5} M) was added to 77 µL of centrifuged saliva in 1 cm path length disposable microcuvettes and mixed. The samples were kept in the dark for 30 min at room temperature and the absorption was then measured at 517 nm (A_c) using methanol as blank. The absorbance of methanolic solution of $DPPH^{\circ}$ was also measured as control (A_c). All experiments were carried out in at least duplicate and radical scavenging activity was calculated using the following relationship:

DPPH radical scavenging activity (%) = $[(A_c - A_S)/A_c] \times 100$

Antioxidant activity in saliva by FRAP

The antioxidant activity of salivary samples was determined by ferric reducing power (FRAP) assay [16]. The FRAP reagent was prepared by mixing 300 mM acetate buffer

pH 3.6, with 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM Fe_3Cl (volume ratios of 1/10/1 respectively). The freshly prepared FRAP reagent (250 µL) was warmed to 37 °C for 10 min. 8.5 µL of centrifuged saliva sample and 25 µL deionized water were then added, mixed and absorbance measured at 595 nm after 30 minutes.

A standard curve was prepared using different concentrations (1-12 mM) of $FeSO_4 \cdot 7H_2O$. The results were corrected for dilution and expressed in mmol $FeSO_4$/L.

Determination of uric acid in saliva

The concentration of uric acid in saliva was determined using an enzymatic method described for assay of uric acid in serum [17]. The assay was based on enzymatic production of hydrogen peroxide (H_2O_2) coupled with catalytic oxidation of p-hydroxybenzoate and 4-aminoantipyrine in the presence of peroxidase. The pink chromophore thus formed was then detected at 505 nm.

Determination of salivary ascorbic acid by HPLC

The samples were also investigated by HPLC at constant temperature using a modification a specific method described by Meyle [18]. In practice, saliva samples and standards were quantified simultaneously by HPLC with Diode Array Detector (HPLC-DAD). The HPLC-DAD system consisted of an Agilent 1200 Series system (Agilent Technologies) composed of a G1379A degasser, a G1311A quaternary pump and a G1316A column oven set at 30°C. The HPLC column was Zorbax Eclipse plus C8 (250 x 4.6 mm, 5 µm) with a flow rate of 1 mL/min. This was coupled to a G1315B photodiode-array detector (PAD) set to scan from 190 to 400 nm. Analysis was performed using the following gradient elution: solvent A was methanol/0.1% TFA (15/85); Solvent B methanol/0.1% TFA (85/15). The gradient was started immediately upon injection. The 80% A/20% B hold for 6 min, followed by raising the percent of B to 60% during the next 13 min. Each run was followed by a 10 min wash with 85% acetonitrile. Before injection of the next sample, the column was equilibrated with mobile phase A for 10 min. Each sample was filtered by a 0.2 mm filter and injected 20 µL each time. The flow rate was kept constant at 1.0 mL/min and the column temperature was set at 30°C. Various concentrations of pure ascorbic acid were used as internal standard.

Statistical analysis

Each assay was repeated at least duplicate and the results were presented as mean ± SD values. Statistical difference between individuals was compared by un-paired t-test, p values less than 0.05 were retained as significant. Significant differences between means were determined by Duncan's multiple range tests. *P* values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSIONS

Salivary flow rate

Table 1 has compared the results of flow rates in smokers and non smokers. Salivary flow rates were expressed as volume of un-stimulated saliva (ml) collected per minute. Statistically significant changes in saliva flow rate were observed between smokers and non-smokers. It was found that the saliva flow rate ranged from 0.9 to 1.50 $ml.min^{-1}$ in non-smokers with a significantly decrease to (0.6-1.1 $ml.min^{-1}$) among smokers ($p<0.05$). This finding supports our previous results obtained for un-stimulated salivary flow rate in passive smokers [19]. It has been found that the rate of flow of any biological fluid such as tears [20-22] and saliva [23-26], depends on various external and internal factors. In our previous studies, we had found that the salivary flow rate does not significantly alter due to exercise intensity [10]. However, it is suggested that

the decrease in salivary volume and rate of flow in smokers obtained from the present study is due to the presence of various toxic chemicals in cigarette smoke. It is not surprising that many heavy smokers complain from dry mouth even at young ages.

DPPH test

The radical scavenging activity of saliva samples against stable DPPH radical was determined by following the change in color of $DPPH^{\cdot}$ as the result of its reduction by antioxidants present in saliva. It was expressed as % of scavenging activity against DPPH radical. Table 2 has compared the radical scavenging activity of saliva against $DPPH^{\cdot}$ in smokers and non-smokers. It was observed that activity of saliva against free radicals such as $DPPH^{\cdot}$ was significantly decreased in smokers (about 25%). Human saliva is the first biological fluid that encounters the cigarette smoke. Therefore, its main important duty must be their reduction by antioxidant action.

It is known that cigarette smoke is composed of a tar phase and a gas phase; both composed of considerable number of different free radicals as well as non-radical oxidants [27]. The free radicals are able to initiate the generation of various reactive oxygen species (ROS). These species can then increase and promote oxidative damage to the oral cavity, respiratory and gastrointestinal tracts.

FRAP test

The FRAP test was used for quantification of the general capacity of saliva to chelate and inactivate metal ions. The FRAP test especially measures ferric and ferrous ions which are involved in the formation of highly reactive oxygen and nitrogen species (ROSs and RNSs). Table 3 shows that the reducing power of saliva against ferric ions has significantly decreased in salivary fluid of smokers. The FRAP test is a sensitive and accurate test for measuring salivary antioxidant power. The results obtained in this part of study supports our finding for peroxidase, the antioxidant enzyme of saliva [28] and DPPH assay in other sections of the present work.

Salivary uric acid

The salivary antioxidant system is composed of various molecules and enzymes. The most important antioxidants in human saliva are water soluble uric acid, peroxidase and ascorbic acid respectively. The concentration of lipid-soluble antioxidants is very low, contributing about 10% of the total salivary antioxidant capacity [29-31]. It has been reported that the uric is responsible for about 70% of the total salivary antioxidant capacity [30]. The present study showed that

Table 1. Salivary flow rate of smokers compared to non-smoker group. Each value is the mean obtained from at least 50 measurements (25 cases each duplicate)

Flow rate (mL/mim)	Smokers (n=25)	Non-smokers (n=25)
Mean	0.85	1.10
Range	0.6-1.1	0.9-1.5
Standard deviation (SD)	0.50	0.66

Table 2. Antioxidant activity in saliva of smokers and non-smokers examined by $DPPH^{\cdot}$ and FRAP tests. Each value is the mean obtained from at least 50 measurements (duplicate tests for 25 cases).

	% Scavenging activity by DPPH	FRAP (μmol Fe/min ml)
Smokers	0.38 ± 0.08	0.18 ± 0.06
Non-smokers	0.47 ± 0.12	0.36 ± 0.09
p values	<0.01	<0.01

salivary uric acid was significantly decreased in smokers when compared to the control group, 6.32 compared to 5.37 mg/dl (Table 3). The amount of uric acid in un-stimulated saliva of normal subjects obtained in this research is higher than the reported value (1.75 ± 0.6 mg/dl) in stimulated saliva [29]. However in reference to smokers, these results somewhat correlate with the findings of [31] who examined the resting and stimulated saliva in terms of their origin and uric acid content. They showed that among other antioxidants, uric acid was significantly higher in un-stimulated saliva samples regardless of their origin. It is worth to remember that uric acid is the most important hydrophilic antioxidant in human saliva. The molecule plays a dual action being both a chelating agent to prevent the attack of free radicals and a scavenger of already produced free radicals. Therefore, its reduction in the oral cavity can be highly crucial in diminishing the antioxidant power of saliva.

Table 3. Concentrations of uric acid and ascorbic acid, the two most important non-enzymatic antioxidants, in saliva samples. The values are mean obtained from at least 50 measurements.

	Uric acid (mg/dl)	Ascorbic acid (μg/ml)
Smokers	5.37 ± 0.22	380 ± 15.50
Non-smokers	6.32 ± 0.45	450 ± 22.80
p values	<0.03	<0.02

Salivary ascorbic acid

Ascorbic acid is the second most important non-enzymatic antioxidant in salivary fluid [31]. HPLC analysis of vitamin C was performed on mixed samples of saliva from smokers and non-smokers. Figure 1 represents the typical HPLC chromatogram for smokers (A) and non-smokers (B). The HPLC results showed that concentration of ascorbic acid in saliva samples was 380-478 μg/mL and 395-289 μg/mL for smokers and non-smokers respectively. The average values were 380 μg/mL (S.D±15.50) and 450 μg/mL (S.D± 22.80) respectively (Table 3). It has been shown that the rate of secretion of the ascorbic acid depends on the gland secreting saliva [32].

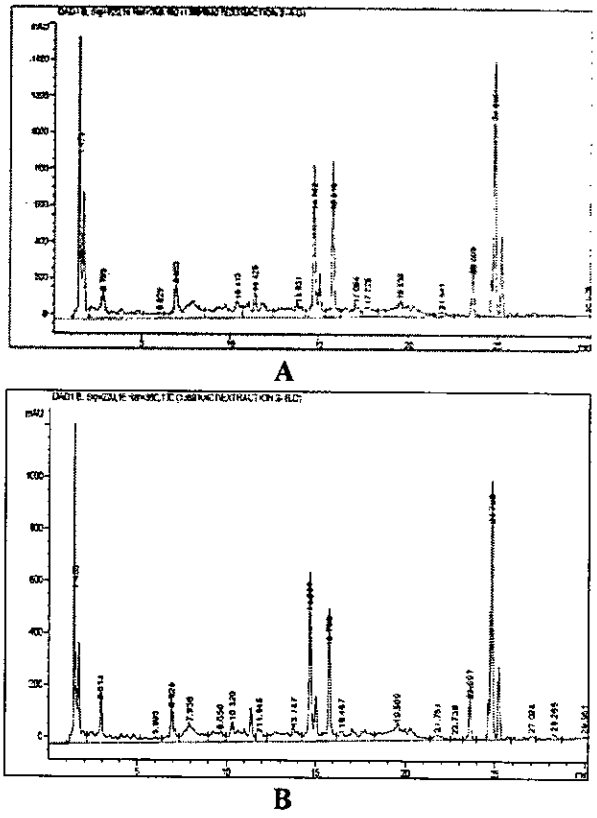

Fig. 1. HPLC chromatograms obtained from saliva of smokers (A) and non-smokers (B) The peak appeared at 11.30 minutes are related to ascorbic acid.

CONCLUSIONS

It has been demonstrated that smoking of even one cigarette can increase concentrations of tobacco metabolites in salivary fluid of both smokers and non-smokers [33]. Main tobacco metabolites are composed of dangerous free radicals that are harmful to both oral and other body tissues. Oral cavity is the first body site that encounters gaseous and particulate products of cigarette smoke. The presence of free radicals in salivary fluid can seriously damage oral environment through reaction with polyunsaturated fatty acids and lipid peroxidation in the membranes. This may lead to several oral inflammatory and degenerative diseases causing neoplastic transformation. It has been demonstrated that both gaseous and particulate phases of

cigarette smoke contains free radicals as well as other chemicals capable of generating free radicals [34]. Natural antioxidants present in healthy saliva can diminish activities of free radicals and protect oral cavity. Eventually, internal body environment can be less damaged by normal action of the antioxidant system in the salivary fluid. Therefore, reduction of salivary antioxidant power observed in smokers may increase individual sensitivity toward tobacco stress.

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REFERENCES

[1] W.A. Pryor, D.G. Prier, D.F. Church, Environ. Health Prospect. 47 (1983) 345.
[2] C.A. O'Neill, B. Halliwell, A. van der Vliet, P.A. Davis, L. Packer, H. Tritschler, W. Storhman, T. Reiland, C.E. Cross, A.Z. Reznick, J. Lab. Clin. Med. 124 (1994) 359.
[3] J.L. Herrera, I.I.M.F. Lyons, L.F. Johnson, J. Clin. Gastero. 10 (1998) 569-578.
[4] I.D. Mandel, J. Clin. Dent. Res. 66 (1987) 623.
[5] A. Bardow, J. Madsen, B. Nauntofte, Clin. Oral Ivest. 4 (2000) 245.
[6] A. Rezaei, R. Sariri, Pharmacologyonline. 1 (2011) 428.
[7] D. Aizenbud, Y. Peri-Front, R.M. Nagler, Arch. Oral Biol. 53 (2008) 517.
[8] S.R. Porter, J.C. Leao, Alim. Pharma. Therap. 21 (2005) 295.
[9] M.J. Novak, R.M. Potter, J. Blodgett, J.L.Ebersole, J. Periodont. 79 (2008) 629.
[10] A. Damirchi, R. Sariri, M. Kiani, V. Jafarian, Euro. J. Appl. Physiol. 3 (2010) 245.
[11] L.A. Tabak, M.J. Levine, I.D. Mandel, L.A. Elison, J. Oral Pathol. 11 (1982) 1.
[12] R. Kohen, O. Tirosh, K. Kopolovich K, Exp. Gerontol. 27 (1992) 161.
[13] R. Nagler, S. Lischnisky, E. Diamond, Arch. Biochem. Biophys. 379 (2000) 229.
[14] B. Zappacosta, S. Persichilli, A. Mordente, Hum. Experi. Toxico. 21 (2002) 7.
[15] S. Bompadre, L. Leone, A. Politi, M. Battino, Free Radic..Res. 38 (2004) 831.
[16] I.F.E. Benzie, J.J. Strain, Anal. Biochem. 239 (1996) 70.
[17] R.C. Trivedi, L. Rebar, E. Berta, L. Stong, Clin. Chem. 24 (1978) 1908.
[18] J. Meyle, K. Kapitza, Arch. Oral Biol. 35 (1990) 319.
[19] A Rezaei, R. Sariri. Pharmacologyonline. 3 (2011) 462.
[20] The epidemiology of dry eye disease: report of the epidemiology subcommittee of the international dry eye workshop (2007), Ocular Surf. 5 (2007) 93.
[21] A.J. Bron, N. Yokoi, E.A. Gaffney, Ocular Surf. 7 (2009) 78.
[22] E.A. Gaffney, J.M. Tiffany, N. Yokoi, A.J. Bron. Prog. Retin. Eye Res. 29 (2010) 59.
[23] R. Simi, L. Walter, Jr. Siqueira,. Inter. J. Pediat. Otorhinolaryng. 70 (2006) 799.
[24] L. Eliasson, D. Birkhed, A. Carlén. Arch. Oral Biol. 54 (2009) 263.
[25] T. Nederfors, B. Nauntofte, S. Twetman., Arch. Oral Biol. 49 (2004) 507.
[26] T. Nederfors, G. Holmström, G. Paulsson, D. Sahlberg, Swed. Dent. J. 26 (2002) 1.
[27] W.A. Pryor, B.J. Hale, P.I. Premovic, D.F. Church, Science 220 (1983) 425.
[28] R. Sariri R, A. Varasteh, A. Erfani Karimzadeh, Z. Heidari, Health. 2 (2010). 347.
[29] E. Meucci, C. Littarru, G. Deli, G. Luciani, L. Tazza, G.P. Littarru, Free Radic. Res. 29 (1998) 376.
[30] S. Mooe, K.A.C. Calder, N.J. Miller, C.A. Rice-Evans, Free Radic. Res. 21 (1994) 417.
[31] RM. Nagler, I. Klein, N. Zarzhevsky, N. Drigues, A.Z. Reznick. Free Radic. Biol. Med. 32 (2002) 268.
[32] E. Mäkilä, P. Kirveskari, Arch. Oral Biol.14 (1969) 1285.
[33] B. Zappacosta, S. Persichilli, P. De Sole, A. Mordenteb, Arch. Oral Biol. 44 (1999) 485.
[34] D.D. Bankson, M. Kenstin, N. Rifai, Clin. Lab. Med. 13 (1993) 463.