

Comparison of Salivary Antioxidants in Healthy Smoking and Non-smoking Men

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Background: Tobacco use is known as a serious global public health problem, and is also an important risk factor for oral diseases. Saliva is the first biological medium encountered during inhalation of cigarette smoke. Therefore, the main aim of this study was to compare the levels of salivary antioxidants between healthy smoking and non-smoking men.

Methods: Unstimulated whole saliva samples were collected from 80 men. Forty subjects were smokers with a daily consumption of 20 cigarettes for at least 10 years and 40 subjects were non-smokers. The salivary levels of uric acid, superoxide dismutase, glutathione peroxidase, and peroxidase were measured and compared between studied groups.

Results: The mean levels of salivary superoxide dismutase, glutathione peroxidase, and peroxidase were significantly lower in smokers than non-smokers. There was no statistically significant difference in the salivary uric acid level between smokers and non-smokers.

Conclusions: Measurement of antioxidant agents in human saliva might be useful for estimating the level of oxidative stress caused by cigarette smoke.
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Key words: saliva, antioxidants, smokers

Tobacco use is known as one of the most important risk factors for oral diseases such as oral cancer, periodontal disease, cleft lip, cleft palate, alveolar bone loss and black hairy tongue.⁽¹⁻⁴⁾ It is noted that cigarette smokers have risks of oral cancer 2 to 5 times that of non-smokers.^(5,6) Tobacco consumption has a direct correlation with 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.^(7,8) Reactive oxygen species, free radicals and reactive nitrogen species in inhaled cigarette

smoke have been suggested to induce a gradually evolving process, initially expressed by dysplastic lesions which then transform into carcinoma lesions.⁽⁹⁾ Recently, it has been demonstrated that the imbalances in free radical levels and reactive oxygen species with antioxidants may play a key role in the onset and development of several inflammatory oral pathologies.⁽¹⁰⁾ This evidence emphasizes the role of cigarette smoking on salivary antioxidants in the pathogenesis of oral cancers.⁽⁹⁾ Cigarette smoke is a major source of free radicals and tobacco smoke contains oxidants and pro-oxidant agents.⁽¹¹⁻¹³⁾

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Oxidative damage to DNA and other macromolecules has been suggested in the pathogenesis of a wide variety of diseases.^(14,15) Free radical formation is naturally controlled by antioxidants. Antioxidants are capable of deactivating or stabilizing free radicals before they injure cells.^(16,17) Antioxidants are present in all body fluids including saliva. Saliva may constitute a first line of defense against oxidative stress and has protective effects against microorganisms, toxins and oxidants.^(10,18) In the second half of the 20th century, it was suggested that saliva could be used in diagnostics. Today saliva is often used to diagnose systemic and local diseases. The main advantage of this medium is the easy, non invasive sampling method compared with that for blood.⁽¹⁹⁾ However the use of saliva for detection of antioxidant alterations in smokers has been little utilized or appreciated. Therefore the aim of this study was to compare the level of salivary antioxidants such as uric acid (UA), superoxide dismutase (SOD), peroxidase (POx) and glutathione peroxidase (GPx) between smoking and non-smoking men.

METHODS

Eighty men (40 non-smokers, age range 30-48 years old, mean age 38 ± 5.3 years and 40 smokers, age range 31-50 years old, mean age 40 ± 4.6 years with a daily consumption of 20 cigarettes for at least 10 years), were enrolled in this study. All subjects were clinically healthy and had moderate periodontitis (clinical attachment loss was 3 to 4 mm).

Participants in this study were selected from the Oral Medicine Department of Hamadan Dental Faculty, Hamadan, Iran during the years 2010 to 2011. Exclusion criteria for both groups were any sign of cardiovascular, endocrine, gastrointestinal, oral and respiratory disease, alcohol consumption, and history of drug treatment or therapy within the three previous months. Before salivary collection, the study was explained and participants were asked to complete an informed consent form.

Salivary collection

In this study unstimulated whole saliva was collected from each subject between 8-10 a.m to avoid circadian variations. No oral stimulus was done for 90 minutes prior to salivary collection. Also, the smoking volunteers were asked not to smoke for one

hour prior to the experiment. We employed a collection protocol using the modified Navazesh method.⁽²⁰⁾ While in a sitting position, the participants were asked to swallow saliva, then stay motionless and allow the saliva to drain passively for 10 minutes over the lower lip into a sterile plastic vial. Saliva samples were immediately centrifuged (1000 g, 10 minutes) at 4°C to remove cell debris. The resulting supernatants were immediately deep-frozen at -80°C and stored for later analysis.

Measurement of Cu/Zn- SOD

The activity of Cu/Zn- SOD was measured using a commercial kit (Ransod kit, Randox Laboratories Ltd, Crumlin, UK). Measurement of the enzyme was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase and reacted with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The formazan was read at 740 nm. One unit of Cu/Zn- SOD was defined as the amount of enzyme necessary to produce 50% inhibition in the INT reduction rate.

Determination of UA The UA concentration was measured in the saliva using a spectrophotometric kit (Parsazmun Co, Tehran, Iran). UA was transformed into allantoin and hydrogen peroxide by uricase. Under the catalytic influence of peroxidase, the chromogen (4-aminophenazone/n-ethyl-methylanilin propan-sulphonate sodium) was oxidized to form a red compound, and the intensity of the produced color was proportional to the amount of UA present in the sample, which was read at 546 nm.

Measurement of GPx The amount of GPx was determined using a commercially available kit (Ransel kit, Randox Laboratories Ltd, Crumlin, UK) by measuring the rate of oxidation of NADPH at 340 nm. A unit of enzymewas expressed as the amount of enzyme needed to oxidize 1 nmol of NADPH oxidase/minute.

Measurement of POx

POx was measured by the method of Pruitt et al.⁽²¹⁾ The assay was performed by mixing 1.0 ml phosphate buffer (pH 7.0), 1.0 ml guaiacol solution and 1.0 ml of a saliva sample. The reaction was started by adding 1.0 ml of H₂O₂ stock solution. Absorbance at 470 nm (A) and time (T) data were monitored. Initial rates (dA/dT) were determined by

linear regression analysis of the recorded data. One unit of POx was defined as the amount that yielded 1.0 dA for one minute. The activity of the enzyme was expressed as units per milligram of protein in saliva. The protein content in the samples was determined using the Bradford method.⁽²²⁾

Statistical analysis

Statistical analysis was performed using SPSS 18 software for Windows: all values were reported as mean \pm SD. The statistical significance of differences in salivary antioxidant levels between smoking and non-smoking men was estimated by t-test. In this study a *p* value less than 0.05 were accepted as significant.

RESULTS

The mean levels of salivary antioxidants showed that the mean GPx activity was 94.54 ± 37.47 U/L in the smoking group, and 133.4 ± 26.59 U/L in the non-smoking group. The level of GPx activity was significantly (*p* = 0.000) lower in the smoking group than the non-smoking group. The mean concentrations of salivary UA were 2.8 ± 2.1 mg/dl and 3.7 ± 2.6 mg/dl in the smoking and non-smoking groups, respectively, with no significant (*p* = 0.094) difference between groups. The mean SOD activity in the smoking and non-smoking groups were 6.24 ± 2.62 and 8.07 ± 1.30 U/ml respectively. The level of SOD was significantly (*p* = 0.000) lower in the smoking than non-smoking group. The mean level POx was 3.67 ± 2.35 U/mg protein in the smoking group and 7.53 ± 6.24 U/mg protein in the non-smoking group, with a significantly (*p* = 0.036) lower level in the smoking group.

DISCUSSION

Cigarette smoke is a mixture of chemicals containing over 4000 constituents. Some of the compounds identified include nicotine, ammonia, acrolein, phenols, acetaldehyde, benzopyrine, nitrogen oxides, carbon monoxide, polonium, radium and thorium.⁽²³⁾ In addition, cigarette smoke contains free radicals that can cause cellular damage. It was demonstrated that one cigarette puff contains 1014 free radicals in the tar phase and 1015 radicals in the gas phase, (the tar and gas phases are two major

phases in cigarette smoke).⁽²⁴⁾ Pryor and Stone showed the relationship between DNA damage and tar phase free radicals after smoking.⁽²⁵⁾ These radicals are mostly quinone-hydroquinone and they are not highly reactive.⁽²⁵⁾ On the other hand, gas phase free radicals are generally more reactive.⁽²⁶⁾ Tobacco smoke can alter the antioxidative capability of saliva, but the causes of these changes are not exactly known.⁽²⁷⁾ Also, it has been noted that the salivary antioxidant defense system, appears to be less efficient with age. However, Nagler studied the antioxidant profile of human saliva and obtained the following values for unstimulated whole saliva (WS) and parotid saliva (PS) in healthy non-smokers as follows: POx (WS: 283.9 & PS: 611 mU/ml), SOD (WS: 0.79 & PS: 0.80 U/ml), UA (WS: 2.87 & PS: 10.5 mg/dl), sulfhydryls (WS: 26.6 & PS: 23.67 μ M), lysozyme (WS: 28,11 & PS: 27.18 mg/L) and total antioxidant status (WS: 0.68 & PS: 0.87 mmol/L).⁽¹⁸⁾ In our study the mean level of salivary GPx activity was significantly lower in the smoker than non-smoker group. In agreement with this finding Zappacosta et al. showed smoking of a single cigarette induced a significant reduction in the salivary glutathione concentration.⁽²⁸⁾

Kanehira et al, demonstrated that the activity level of salivary glutathione and POx were significantly higher in non-smokers than smokers.⁽²⁹⁾ In contrast, Sohn et al demonstrated that the activity of glutathione was significantly increased in rats exposed to cigarette smoke.⁽³⁰⁾ On the other hand, studies by Kocyigit et al showed erythrocyte glutathione activity was lower in smokers than non-smokers.⁽³¹⁾ Also, Diken et al, reported that smoking did not affect the activity of glutathione in erythrocytes.⁽³²⁾ The conflicting results in these studies might arise from differences in smoking patterns, the numbers and ages of samples, the type of tobacco, the cigarette design including filtration, blend selection, paper and additives and the structure of the studies (in-vivo, in-vitro or animal study).

In this study, the uric acid concentration was lower in smokers than non-smokers but this difference was not statistically significant. Uric acid is one of the most important antioxidants and contributes approximately 70% of the total salivary antioxidant capacity.⁽¹⁸⁾ This finding is in disagreement with Zappacosta et al.⁽²⁸⁾ In contrast, Greabu et al. concluded that exposure to cigarette smoke caused a sig-

nificant decrease in salivary uric acid and amylase.⁽³³⁾ Tsuchiya et al. also demonstrated that smoking a single cigarette rapidly reduces the concentration of plasma antioxidants such as uric acid.⁽³⁴⁾ According to our results, the mean level of salivary SOD activity was significantly lower in the smoker group than non-smokers. This finding is in disagreement with Kanehira et al.⁽²⁹⁾ This difference may be related to the age of the subjects who were evaluated for salivary antioxidant status. Agnihotri et al., showed that the mean levels of SOD activity in the gingival crevicular fluid (GCF) and saliva of smokers were decreased compared with the control group and the mean levels of SOD in the GCF and saliva of heavy smokers were lower than those in light smokers.⁽³⁵⁾

In the present study the mean level of oral POx was lower in smokers than non-smokers. Reznick et al. in in-vivo and in-vitro studies, showed a sharp drop of oral peroxidase activity in smokers and non-smokers after smoking a single cigarette.⁽³⁶⁾ Klein et al. reported that exposure of the saliva of non-smoking subjects to gas phase cigarette smoke caused 76% loss of oral peroxidase activity.⁽³⁷⁾ Also, Gio et al. demonstrated the IgA concentration, amylase activity and oral peroxidase activity were greater in non-smokers than smokers.⁽³⁸⁾ Oral peroxidase is a very important salivary antioxidant and is composed of two enzymes, salivary peroxidase, which contributes 80% of oral peroxidase, and myeloperoxidase, which contributes the main 20% of oral peroxidase.⁽³⁶⁾

It is noted that cyanide is one of the most important factors responsible for cigarette smoke-associated loss of oral peroxidase activity.⁽³⁸⁾ Also, most of the time, the oral epithelium of smokers is unprotected by oral peroxidase against the negative effects of thiocyanate ions and hydroxyl radicals produced by hydrogen peroxide.⁽³⁶⁾ The finding of reduced oral peroxidase levels in smoking subjects may represent a contributory mechanism for initiation and progression of cigarette smoke-related oral diseases such as oral cancer. In 2010, Goku et al. evaluated the oxidant-antioxidant status of blood samples and tumor tissue in patients with oral squamous cell carcinoma and reported that antioxidant levels were significantly reduced in tissue samples from these patients compared with the control group.⁽³⁹⁾

Conclusion

The results of this study suggest that cigarette smoke is associated with a significant decrease in salivary antioxidant concentrations.

REFERENCES

1. Khalili J. Oral cancer: Risk factors, prevention and diagnostic. *Exp Oncol* 2008;30:259-64.
2. International Agency for Research on Cancer: WHO. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Betel-quid and Areca-nut Chewing and Some Areca-nut-derived Nitrosamines. Vol. 85. Lyon, France: IARC, 2004.
3. Banoczy J, Gintner Z, Dombi C. Tobacco use and oral leukoplakia. *J Dent Educ* 2001;65:322-7.
4. Meraw SJ, Mustapha IZ, Rogers RS. Cigarette smoking and oral lesions other than cancer. *Clin Dermatol* 1998;16:625-31.
5. Blot WJ, Mclaughlin JK, Winn DM, Austin DF, Greenberg RS, Preston-Martin S, Bernstein L, Schoenberg JB, Stemhagen A, Fraumeni JF Jr. Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res* 1988;48:3282-7.
6. Hayes RB, Bravo-Otero E, Kleinman DV, Brown LM, Fraumeni JF, Harty LC, Winn DM. Tobacco and alcohol use and oral cancer in Puerto Rico. *Cancer Causes Control* 1999;10:27-33.
7. Khor GH, Siar CH, Jusoff K. Chromosome 17 aberration of oral squamous cell carcinoma in Malaysia. *Global J Health Sci* 2009;1:150-6.
8. Sharma SM, Mohan M, Kumari S, Sorake SH. Evaluation of glutathione in oral squamous cell carcinoma. *J Maxillofac Oral Surg* 2009;8:270-4.
9. Hasnis E, Reznick AZ, Pollack S, Klein Y, Nagler RM. Synergistic effect of cigarette smoke and saliva on lymphocytes—the mediatory role of volatile aldehydes and redox active iron and the possible implications for oral cancer. *Int J Biochem Cell Biol* 2004;36:826-39.
10. Battino M, Ferreiro MS, Gallardo I, Newman HN, Bullon P. The antioxidant capacity of saliva. *J Clin Periodontol* 2002;29:189-94.
11. Pasupathi P, Rao YY, Farook J, Saravanan G, Bakthavathsalam G. Effect of cigarette smoking on lipids and oxidative stress biomarkers in patients with acute myocardial infarction. *Res J Medicine & Med Sci* 2009;4:151-9.
12. Preston AM. Cigarette smoking-nutritional implications. *Prog Food Nutr Sci* 1991;15:183-217.
13. Kosecika M, Erelb O, Sevincc E, Selekb S. Increased oxidative stress in children exposed to passive smoking. *Int J Cardiol* 2005;100:61-4.

14. Krajcovicova-Kudlackova M, Valachovicova M, Paukova V, Dusinska M. Effects of diet and age on oxidative damage products in healthy subjects. *Physiol Res* 2008;57: 647-51.
15. Salmon TB, Evert BA, Song B, Doetsch PW. Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 2004;32: 3712-23.
16. Rahman K. Studies on free radicals, antioxidants, and cofactors. *Clin Interv Aging* 2007;2:219-36.
17. Sapakal VD, Shikalgar TS, Ghadge RV, Adnaik RS, Naikwade NS, Magdum CS. In vivo screening of antioxidant profile: A review. *J Herb Med Toxicol* 2008;2:1-8.
18. Nagler RM, Klein I, Zarzhevsky N, Drigues N, Rezinck A. Characterization of the differentiated antioxidant profile of human saliva. *Free Radic Biol Med* 2002;32:268-77.
19. Pink R, Simek J, Vondrakova J, Faber E, Michl P, Pazdera J, Indrak K. Saliva as a diagnostic medium. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2009;153: 103-10.
20. Navazesh M. Methods for collecting saliva. *Ann NY Acad Sci* 2006;694:72-7.
21. Kruger NJ. The Bradford method for protein quantitation. In: Walker JM, ed *The Protein Protocols Handbook*. New Jersey: Humana Press, 1996:15-20.
22. Pruitt KM, Kamau DN, Miller K, Mansson-Rahemtulla B, Rahmetulla F. Quantitative, standardized assays for determining the concentrations of bovine lactoperoxidase, human salivary peroxidase and human myeloperoxidase. *Anal Biochem* 1990;191:278-86.
23. Pasupathi P, Saravanan G, Farook J. Oxidative stress biomarkers and antioxidant status in cigarette smokers compared to nonsmokers. *J Pharm Sci & Res* 2009;1:55-62.
24. Pryor WA, Hales BJ, Premovic PI, Church DF. The radicals in cigarette tar: their nature and suggested physiological implications. *Science* 1983;220:425-7.
25. Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann NY Acad Sci* 1993;868:12-27.
26. Nabil M, Bendich A. Dietary antioxidants: potential effects on oxidative products in cigarette smoke. *Nutrition Research* 2001;21:551-67.
27. Yildiz L, Kayaoğlu N, Aksoy H. The changes of superoxide dismutase, catalase and glutathione peroxidase activities in erythrocytes of active and passive smokers. *Clin Chem Lab Med* 2002;40:612-5.
28. Zappacosta B, Persichilli S, De Sole P, Mordente A, Giardina B. Effect of smoking one cigarette on antioxidant metabolites in the saliva of healthy smokers. *Arch Oral Biol* 1999;44:485-8.
29. Kanehira T, Shibata K, Kashiwazaki H, Inoue N, Morita M. Comparison of antioxidant enzymes in saliva of elderly smokers and non-smokers. *Gerodontology* 2006;23:38-42.
30. Church DF, Pryor WA. Free radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 1985;64:111-26.
31. Kocyigit A, Erel O, Gur S. Effects of tobacco smoking on plasma selenium, zinc, copper and iron concentrations and related antioxidative enzyme activities. *Clin Biochem* 2001;34:629-33.
32. Diken H, Kelle M, Tumer C, Deniz B, Baylan Y, Sermet A. Effects of cigarette smoking on blood antioxidant status in short-term and long-term smokers. *Turk J Med Sci* 2001;31:553-7.
33. Greabu M, Battino M, Totan A, Mohora M, Mitrea N, Totan C, Spinu T, Didilescu A. Effect of gas phase and particulate phase of cigarette smoke on salivary antioxidants. What can be the role of vitamin C and pyridoxine? *Pharmacol Rep* 2007;59:613-8.
34. Tsuchiya M, Asada A, Kasahara E, Sato EF, Shindo M, Inoue M. Smoking a single cigarette rapidly reduces combined concentrations of nitrate and nitrite and concentrations of antioxidants in plasma. *Circulation* 2002;105: 1155-7.
35. Agnihotri R, Pandurang P, Kamath SU, Goyal R, Ballal S, Shanbhogue AY, Kamath U, Bhat GS, Bhat KM. Association of cigarette smoking with superoxide dismutase enzyme levels in subjects with chronic periodontitis. *J Periodontol* 2009;80:657-62.
36. Reznick AZ, Klein I, Eiserich JP, Cross CE, Nagler RM. Inhibition of oral peroxidase activity by cigarette smoke: In vivo and in vitro studies. *Free Radic Biol Med* 2003;34:377-84.
37. Klein I, Nagler RM, Toffler R, van Der Vliet A, Reznick AZ. Effect of cigarette smoke on oral peroxidase activity in human saliva: role of hydrogen cyanid. *Free Radic Biol Med* 2003;35:1448-52.
38. Goi N, Hirai Y, Harada H, Ikari A, Ono T, Kinase N. Comparison of peroxidase response to mental arithmetic stress in saliva of smokers and non-smokers. *J Toxicol Sci* 2007;32:121-7.
39. Goku S, Patil VS, Jaikhani R, Hallikeri K, Kattappagari KK. Oxidant-antioxidant status in blood and tumor tissue of oral squamous cell carcinoma patients. *Oral Dis* 2010;16:29-33.