Salivary Analysis in Oral Cancer Patients

DNA and Protein Oxidation, Reactive Nitrogen Species, and Antioxidant Profile

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BACKGROUND. Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which induce oxidative and nitrative stress, are main contributors to oral carcinogenesis. The RNS (nitrosamines: nitrates, NO_3, and nitrites, NO_2) are also produced by the reaction of ROS and other free radicals with nitric oxide (NO) and are therefore in equilibrium with it.

METHODS. Whole saliva was collected from a group of 25 consenting oral squamous cell carcinoma (OSCC) patients and from a control group of 25 healthy age- and gender-matched individuals. General and specific salivary antioxidant components, salivary nitrosamines, and oxidatively damaged salivary DNA and proteins were measured.

RESULTS. The findings showed that oxidative and nitrative stress altered the salivary composition in OSCC patients. Analyzed salivary RNS were substantially higher (NO, 60%; NO_2, 190%; NO_3, 93%), whereas all salivary antioxidants were substantially reduced. The 8-hydroxy-deoxyguanosine (8-OHdG) marker (a widely used indicator of DNA oxidation) increased by 65% and the salivary carbonylation level was significantly higher.

CONCLUSION. The increase in ROS and RNS may have been the event that led to the consumption and reduction of salivary antioxidant systems, thus explaining the oxidative damage to the DNA and proteins, and possibly the promotion of OSCC. The oxidized proteins and DNA found in the saliva of the cancer patients seems to be the first demonstration of a direct link between salivary free radicals, antioxidants, and OSCC. This may be important for better understanding the pathogenesis of the disease and may contribute to its diagnosis and treatment. ***Cancer* 2007;109:54–9.** © *2006 American Cancer Society.*

KEYWORDS: saliva, oral, tongue, cancer, antioxidants, ROS, RNS, DNA, 8-OHdG.

Oral squamous cell carcinoma (OSCC) is the sixth most common human cancer, with an increasing incidence in younger people, a high morbidity rate, and a 5-year mortality rate of about 50%.[1–6] Free radicals, such as reactive oxygen and nitrogen species (ROS and RNS), which induce oxidative and nitrative stress, are principal inducers of OSCC. Ma et al.[7] recently demonstrated that oxidative and nitrative stress contribute to the development of oral carcinogenesis from leukoplakia through DNA damage. RNS in the form of nitrosamines (NO_3 and NO_2) and ROS such as superoxide radicals ($O_2^{\bullet}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2), play a key role in human cancer development because they can cause DNA base alterations, strand breaks, damaged tumor suppressor genes, and an enhanced expression of protooncogenes. ROS-induced mutation could also result from protein damage.[8,9] Salivary nitrosamine production and metabolism are also based on the die-

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tary nitrates (NO_3), which are absorbed from the upper gastrointestinal tract and actively concentrated from the plasma into the saliva by the salivary glands through an active transport system similar to that for iodide, thiocyanate, and perchlorate.[10] In the oral cavity the salivary nitrates are turned into nitrites (NO_2), which are of special importance as carcinogenesis promoters because they react with amines and amides to form the carcinogenic nitrosamines.[11,12]

The OSCC-inducing ROS and RNS originate mainly from smoking, alcohol, food, drink, and/or various other volatile sources, which enter freely into the oral cavity through the largest open gate of the body, the mouth. Accordingly, it is of no surprise that evolution armed the oral cavity with an advanced salivary antioxidant system that also contains antinitrosamine inhibitory agents.[13] This salivary antioxidant system is based on enzymatic and nonenzymatic components including peroxidase and superoxide dismutase (SOD) enzymes as well as uric acid (UA) molecules.[14] It also includes another pivotal anticancer salivary enzyme, glutathione S-transferase (GST), which catalyzes glutathione conjugation to the carcinogen electrophilic epoxide intermediates to protect against DNA damage and adduct formation.[15]

We recently suggested that saliva plays a pivotal role in OSCC pathogenesis.[16] This is also supported by Wu et al.,[17] who recently demonstrated that saliva plays an important role in cigarette-related nicotine-induced DNA damage. However, the aforementioned studies did not show salivary involvement directly (by DNA analysis for example) nor did they deal with the in vivo state. Moreover, salivary antioxidants were not evaluated nor were salivary nitrosation products examined in OSCC patients.

The purpose of the current study was to evaluate various oxidative stress-related parameters and the antioxidant profile of the saliva in OSCC patients. This analysis may well be of great importance for further understanding the relation between saliva and free radicals as related to oral cancer pathogenesis.

MATERIALS AND METHODS

Patients

Whole saliva was collected from a group of 25 otherwise healthy and consenting OSCC patients (13 women and 12 men). The patients' mean age was 68 ± 17 years (range, 30–86) and they were compared with a control group of 25 healthy, nonsmoking and nondrinking individuals matched for age and gender. Only 2 of the patients had a history of smoking and none of drinking alcohol. None had a history of oral premalignant lesions; all OSCC lesions were located at the lateral aspect of the mobile tongue.

Saliva Collection

Whole saliva was collected shortly after the diagnosis and before administration of definitive therapy under resting conditions in a quiet room between 8 AM and noon, at least 1 hour after food intake. Patients were asked to generate saliva in their mouths and to spit into a wide test tube for 10 minutes, as previously described.[16] After collection, the saliva was immediately centrifuged at 800*g* at 4°C for 10 minutes and the resulting supernatant was used for further biochemical analysis.

Salivary Antioxidant Analysis

Peroxidase

Peroxidase activity was measured both in the patients' serum and saliva according to the nitrobenzoic acid (NBS) assay as previously described.[14] Briefly, the calorimetric change induced by the reaction between the enzyme and the substrate, dithiobis 2-nitrobenzoic acid (DTNB), in the presence of mercapto-ethanol, was read at a wavelength of 412 nm for 20 seconds.

Glutathione S-transferase (GST)

The GST analysis was performed as previously described.[18] Briefly, an enzyme-immunoassay (EIA) was employed allowing the quantitative determination of the human GST. The enzyme was first coated to the surface of microtiter plates followed by a blocking step and preincubation of the calibrators and samples with a polyclonal rabbit antibody. The GST in the controls and samples then competed with the GST on the plate for antibody binding. After washing, detection of the bound rabbit antibody was performed by peroxidase-labeled goat antirabbit antibody. The amount of converted substrate, indirectly proportional to the amount of GST antigen in the sample, was photometrically determined at 450 nm.

Superoxide dismutase (SOD)

Total activity of SOD isoenzymes (Cu/Zn-SOD and Mn-SOD) was measured using the Xanthine Oxidase/XTT method. That is, a spectrophotometric assay for SOD based on tetrazolium salt 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzenesulfonic acid hydrate reduction by xanthine/xanthine oxidase. The method is a modification of the NBT assay. XTT is reduced by the superoxide anion ($O_2^{\bullet -}$) generated by xanthine oxidase. Formazan is read at 470 nm. SOD inhibits this reaction by scavenging the $O_2^{\bullet}$. One unit of the enzyme is

defined as the amount of enzyme needed for 50% inhibition of absorption in the absence of the enzyme.[14]

Uric acid (UA) concentration

Uric acid concentration was measured with a kit supplied by Sentinel CH (Milan, Italy) as previously described.[14] In the assay, UA is transformed by uricase into allantoin and hydrogen peroxide, which, under the catalytic influence of peroxidase, oxidizes the chromogen (4-aminophenazone/N-ethyl-methyl-anilin propan-sulphonate sodic) to form a red compound whose intensity of color is proportional to the amount of UA present in the sample and is read at a wavelength of 546 nm.

Total antioxidant status (TAS)

The assay used was based on a commercial kit supplied by Randox (Oceanside, CA) in which metmyoglobin in the presence of iron is turned into ferrylmyoglobin. Incubation of the latter with the Randox reagent ABTS results in the formation of a blue-green colored radical that can be detected at 600 nm.[14]

Antioxidant capacity (ImAnOx)

An enzyme-linked immunosorbent assay (ELISA) colorimetric test system (Immundiagnostik, Bensheim, Germany) for the determination of the overall antioxidative capacity of the oral cavity was performed by the reaction of antioxidants in saliva with a defined amount of exogenously provided H_2O_2. The antioxidants in the saliva sample eliminated a certain amount of the hydrogen peroxide provided. The residual H_2O_2 was determined colorimetrically by an enzymatic reaction that involves the conversion of TMB to a colored product. After the addition of a stop solution, the samples were measured at 450 nm in a microtiter plate reader. The quantification was performed with a calibrator. The difference between the applied and measured concentration in a defined time is proportional to the reactivity of the antioxidants of the sample (antioxidant capacity).

Salivary Nitrogen Species Analysis

Salivary nitric oxide (NO) was measured in terms of its products, nitrite (NO_2) and nitrate ($NO_{3)}$, by the Griess method modified by Fiddler[19] using the Nitric Oxide and the Total Nitric Oxide assays kits of Assay Designs (Ann Arbor, MI). This method is based on a 2-step process: the first step is the conversion of nitrate to nitrite using tin metal powder and the second is the addition of sulfanilamide and N (naphthyl) ethylenediamine (Griess reagent). This converts nitrite into a deep purple azo compound, which was measured colorimetrically at 540 nm.

DNA Analysis of Salivary 8-Hydroxy-deoxyguanosine (8-OHdG)

Quantitative measurement of the oxidative DNA adduct 8-OHdG was performed according to the method described by Toyokuni et al.[20] Briefly, the saliva samples were centrifuged at 10,000*g* for 10 minutes and the supernatant was used to determine 8-OHdG levels with a competitive ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan). The determination range was 0.5–200 ng/mL.

Salivary Carbonyls

Salivary carbonyls were analyzed by Western blot for both the healthy and OSCC groups being performed with Oxyblot Kit S-71250 (Intergen, Purchase, NY) using specific anti-dinitrophenyhydrazine (DNPH) antibodies. Between 25 and 30 mL of saliva supernatant was applied to each well, corresponding to 60 mg of protein. Finally, saliva proteins were run on 10% SDS-PAGE (polyacrylamide gel electrophoresis) as previously described by Nagler et al.[21]

Statistical Analysis

For categorical variables, frequencies, percentages, and distribution were calculated. For continuous variables, ranges and medians were calculated. Due to the large inborn variability of parameters in saliva and in line with common practice,[22] median values were calculated and, as small sample size groups were analyzed (fewer then 30 individuals in a group), nonparametric statistical tests were used. Distributions of categorical variables were compared and analyzed by the Fisher-Irwin exact test. The medians between subgroups of patients were compared with a Wilcoxon rank-sum test (pairs of subgroups). The correlation between the parameter levels in patients and controls were analyzed with the Spearman correlation.

RESULTS

Antioxidant Analysis

Both ImAnOx and TAS assays evaluating the general antioxidant capacity of the saliva showed substantially reduced values in the OSCC patients as compared with controls (Fig. 1). The ImAnOx assay revealed a significantly reduced antioxidant capacity by 22% ($P < .05$), from 320 μmol/L to 251 μmol/L, whereas the TAS assay revealed a 49% reduction, from 0.49 mmol/L to 0.25 mmol/L ($P < .05$).

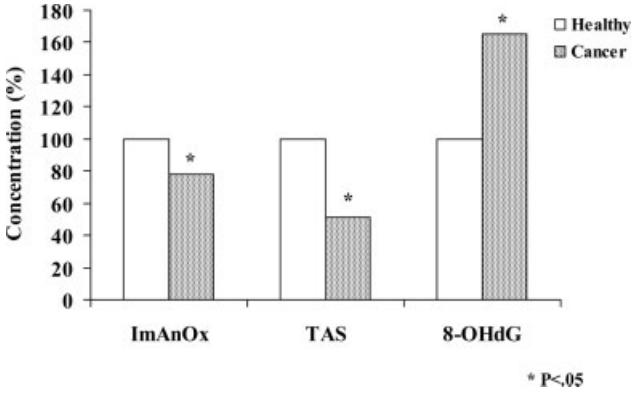

FIGURE 1. Salivary concentrations of general antioxidants: total antioxidant status (TAS) and antioxidant capacity (ImAnOx) and of oxidized DNA (8-OHdG) in healthy (open bars, n = 25) and oral squamous cell carcinoma (OSCC) patients (dotted bars, n = 25). Statistical significance: $*P \leq .05$.

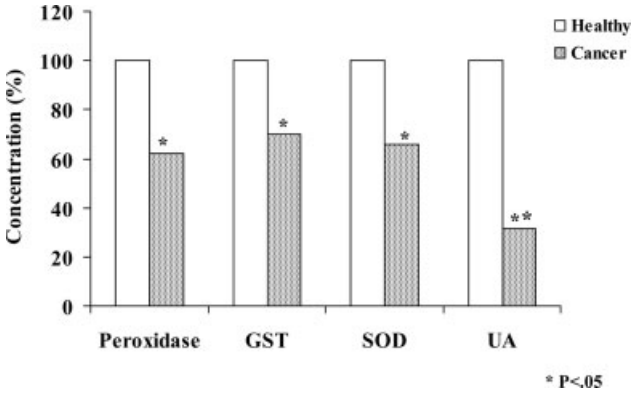

FIGURE 2. Salivary concentrations of specific antioxidants: peroxidase, glutathione S-transferase (GST), and uric acid (UA) and activity levels of superoxide dismutase (SOD) in healthy (open bars, n = 25) and oral squamous cell carcinoma (OSCC) patients (dotted bars, n = 25). Statistical significance: $*P \leq .05$.

Similarly, the salivary-specific antioxidants analyzed—peroxidase, GST, and SOD enzymes and the UA molecule—were reduced by 38% ($P < .05$), 30% ($P < .05$), 34% ($P < .05$), and 69% ($P < .01$), respectively, from 386 mU/mL to 280 mU/mL, from 230 ng/mL to 161 ng/mL, from 1.25 U/mL to 0.90 U/mL, and from 4.12 mg/mL to 1.30 mg/mL (Fig. 2). Indeed, the Spearman correlation coefficients among the various analyzed antioxidants were rather high, indicating a similar pattern of reductions. Thus, the correlation coefficients of ImAnOx and peroxidase, ImAnOx and SOD, ImAnOx and UA, ImAnOx and TAS, and TAS and GST were 0.60, 0.55, 0.50, 0.70, and 0.55, respectively (Fig. 3).

Nitrogen Species Analysis

The salivary concentrations of the analyzed RNS: the NO, NO_2, and NO_3 in healthy controls, were 72 μmol/L, 80 μmol/L, and 37.6 μmol/L, respectively. In the OSCC patients these salivary values were higher by 60%, 190%, and 93%, respectively ($P < .05$) (Fig. 4). The Spearman correlation coefficients between the NO and NO_2 and between the NO and NO_3 salivary concentrations were 0.90 and 0.66, respectively.

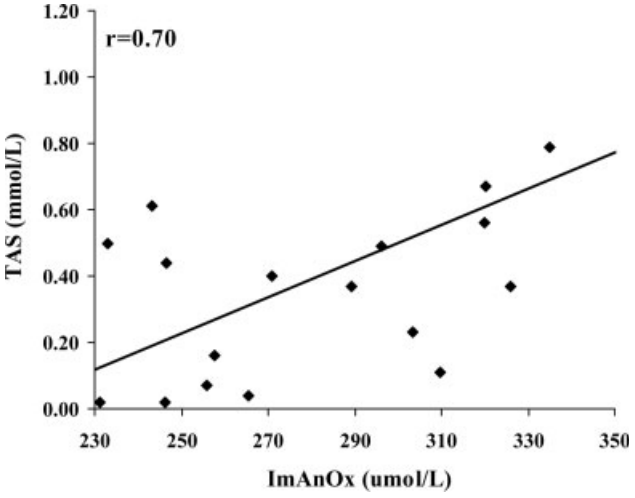

FIGURE 3. Spearman correlation coefficient between salivary general antioxidant assays: total antioxidant status (TAS) and antioxidant capacity (ImAnOx).

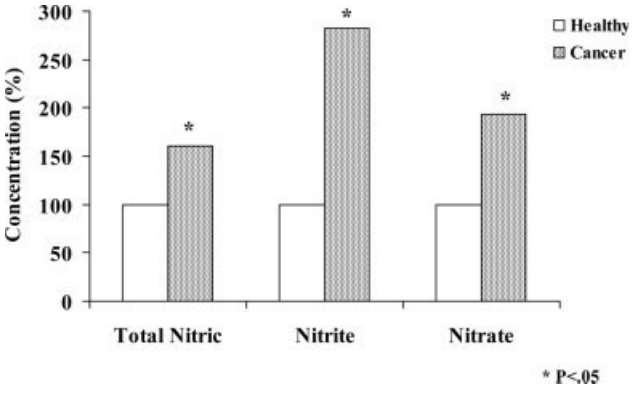

FIGURE 4. Salivary concentrations of total nitric oxide (NO), nitrates (NO_3), and nitrites (NO_2) in healthy (open bars, n = 25) and oral squamous cell carcinoma (OSCC) patients (dotted bars, n = 25). Statistical significance: $*P \leq .05$.

Oxidative DNA and Proteins Analysis

The level of the oxidized DNA as expressed by 8-OHdG levels was increased by 65% ($P < .05$) in the OSCC patients, from 0.68 ng/mL to 1.12 ng/mL (Fig. 1). The Spearman correlation coefficient between the 8-OHdG and ImAnOx was high as well ($r = 0.62$). The Western blot presented in Figure 5 clearly demonstrates the most extensive carbonylation level

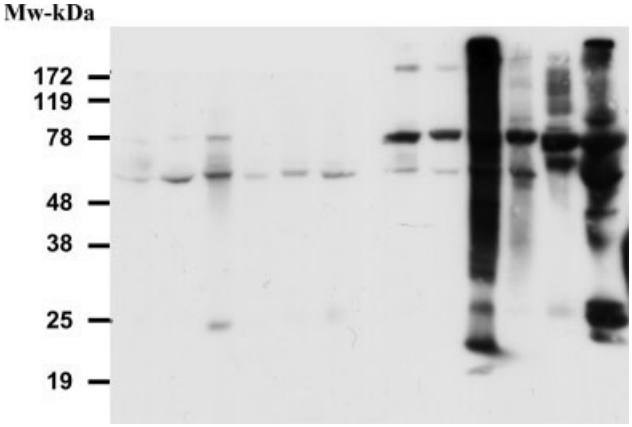

FIGURE 5. Western blot analysis with anti-DNP antibody for salivary protein carbonylation (oxidation level). Lanes 7–12 (right panel) show the increased level of protein carbonyls in oral squamous cell carcinoma (OSCC) patients as compared with the saliva secreted in the healthy controls (lanes 1–6, left panel).

(indication of protein oxidation) in the saliva of OSCC patients as compared with controls.

DISCUSSION

The most interesting and novel finding of the current study was that the salivary composition of OSCC patients is substantially altered with respect to free radical-involved mechanisms. The salivary DNA and proteins in these patients were found to be profoundly oxidized, whereas all salivary RNS analyzed were significantly higher and all salivary antioxidants significantly reduced. Whether the demonstrated increase in RNS (3-fold in nitrites, which are the precursors of carcinogenic nitrosamines) was the event that led to the consumption and reduction of the salivary antioxidant systems remains an open question. The opposite might be true, but in any case, this explains the oxidative damage to the salivary DNA and proteins that may be the event promoting OSCC. The oxidized proteins and DNA found in the patients' saliva seem to be the first direct link demonstrated between salivary free radicals, the antioxidant system, and OSCC, as the salivary DNA is derived from exfoliative oral epithelial cells (in this case, OSCC cells).[21]

The development of cancer is multifactorial, depending on the extent of DNA damage that is proportional to the magnitude of oxidative and nitrative stress. This stress reflects the net effect of both ROS and RNS on the one hand and the effectiveness of antioxidant defense and the DNA repair systems on the other. In fact, it has been found that, whereas ROS and RNS are involved in the initiation and promotion of multistep carcinogenesis, both are inhibited by antioxidants.[23,24] However, when the equilibrium is broken either by a reduction in the levels of antioxidants or by enhancement of ROS and RNS levels, DNA is oxidized and cancer evolves. This is precisely what we observed in the saliva of the OSCC patients in the current study.

Another interesting observation was that nearly all the analyzed OSCC were in patients who belong to the de novo evolving cancer ('genetic') group; that is, without a history of premalignant lesions or of smoking and drinking. Hence, in these patients hereditary predisposition factors are presumably responsible for the OSCC. It is tempting to speculate that in these patients some genetic factors (such as an enhanced salivary transporter of nitrates or overproducing NO synthase [NOS] enzyme) are responsible for the increased levels of salivary RNS observed or perhaps vice versa, reduced activity of the salivary antioxidant enzyme/s or of the transport of UA. In spite of this suggested genetic background, it is also possible that the development of the oral cavity cancer itself, or habits that lead to oral cavity cancer, are responsible for these observed changes in salivary composition. However, in almost all the patients analyzed (all but 2 were nonsmokers), increased salivary RNS/ROS *could not* have originated from exposure to cigarette smoke or to any other known exogenous source, such as secondhand smoking or alcohol. None of the patients consumed alcohol in higher-than-average amounts nor had they been exposed consistently and substantially to passive smoking in their vicinity.

The current study sheds further light on the role of saliva and oxidative stress in the pathogenesis of oral and oropharyngeal cancer as being based on the ever continuous and intimate contact between saliva and the mucosa (from where this cancer evolves). As saliva can be easily harvested and monitored (for its antioxidants, nitrosation products, oxidized DNA, proteins, etc), we have a unique opportunity to evaluate it and perhaps even to intervene, with local therapeutic agents acting as antioxidants that can be easily applied to the mucosa. Moreover, these demonstrated salivary oxidative alterations may be used for diagnostic purposes, as we have recently suggested for other "traditional" tumor markers.[25]

REFERENCES

1. Kantola S, Parikka M, Jokinen K, et al. Prognostic factors in tongue cancer—relative importance of demographic, clinical and histopathological factors. *Br J Cancer*. 2000;83:614–619.

2. Alho OP, Kantola S, Pirkola U, Laara E, Jokinen K, Pukkala E. Cancer of the mobile tongue in Finland—increasing incidence, but improved survival. *Acta Oncol*. 1999;38:1021–1024.
3. Myers JN, Elkins T, Roberts D, Byers RM. Squamous cell carcinoma of the tongue in young adults: increasing incidence and factors that predict treatment outcomes. *Otolaryngol Head Neck Surg*. 2000;122:44–51.
4. Ribeiro KC, Kowalski LP, Latorre MR. Impact of comorbidity, symptoms, and patients' characteristics on the prognosis of oral carcinomas. *Arch Otolaryngol Head Neck Surg*. 2000;126: 1079–1085.
5. Sparano A, Weinstein G, Chalian A, Yodul M, Weber R. Multivariate predictors of occult neck metastasis in early oral tongue cancer. *Otolaryngol Head Neck Surg*. 2004;131:472–476.
6. Hinerman RW, Mendenhall WM, Morris CG, Amdur RJ, Werning JW, Villaret DB. Postoperative irradiation for squamous cell carcinoma of the oral cavity: 35-year experience. *Head Neck*. 2004;26:984–994.
7. Ma N, Tagawa T, Hiraku Y, Murata M, Ding X, Kawanishi S. 8-Nitroguanine formation in oral leukoplakia, a premalignant lesion. *Nitric Oxide*. 2006;14:137–143.
8. Patel RP, McAndrew J, Sellak H, et al. Biological aspects of reactive nitrogen species. *Biochim Biophys Acta*. 1999;1411: 385–400.
9. Liu RH, Hotchkiss JH. Potential genotoxicity of chronically elevated nitric oxide: a review. *Mutat Res*. 1995;339:73–89.
10. Tenovou J. The biochemistry of nitrates, nitrites, nitrosamines and other potential carcinogens in human saliva. *J Oral Pathol*. 1986;15:303–307.
11. Xia DS, Deng DJ, Wang SL. Destruction of parotid glands affects nitrate and nitrite metabolism. *J Dent Res*. 2003; 82:101–105.
12. Deng DJ. Progress of gastric cancer etiology: N-nitrosamides 1999s. *World J Gastroenterol*. 2000;6:613–618.
13. Stich HF, Rosin MP, Bryson L. The inhibitory effect of whole and deproteinized saliva on mutagenicity and clastogenicity resulting from a model nitrosation reaction. *Mutat Res*. 1982;97:283–292.
14. Nagler RM, Klein I, Zarzhevsky N, Drigues N, Reznick AZ. Characterization of the differentiated antioxidant profile of human saliva. *Free Radic Biol Med*. 2002;32:268–277.
15. Sturgis EM, Wei Q. Genetic susceptibility—molecular epidemiology of head and neck cancer. *Curr Opin Oncol*. 2002;14:310–317.
16. Reznick AZ, Hershkovich O, Nagler RM. Saliva—a pivotal player in the pathogenesis of oropharyngeal cancer. *Br J Cancer*. 2004;91:111–118.
17. Wu HJ, Chi CW, Liu TY. Effects of pH on nicotine-induced DNA damage and oxidative stress. *J Toxicol Environ Health A*. 2005;68:1511–1523.
18. Sundberg AG, Appelkvist EL, Backman L, Dallner G. Quantitation of glutathione transferase-pi in the urine by radioimmunoassay. *Nephron*. 1994;66:162–169.
19. Fiddler RN. Collaborative study of modified AOAC method of analysis for nitrite in meat and meat products. *J Assoc Off Anal Chem*. 1977;60:594–599.
20. Toyokuni S, Tanaka T, Hattori Y, et al. Quantitative immunohistochemical determination of 8-hydroxy-2′-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab Invest*. 1997;76:365–374.
21. Nagler RM, Lischinsky S, Diamond E, Klein I, Reznick AZ. New insights into salivary lactate dehydrogenase of human subjects. *J Lab Clin Med*. 2001;137:363–369.
22. Hardt M, Witkiwska HE, Webb S, et al. Assessing the effects of diurnal variation on the composition of human parotid saliva: quantitative analysis of native peptides using iTRAQ reagents. *Anal Chem*. 2005;77:4947–4954.
23. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic Biol Med*. 1990;8:583–599.
24. Oberley LW, Oberley TD. Role of antioxidant enzymes in cell immortalization and transformation. *Mol Cell Biochem*. 1988;84:147–153.
25. Nagler RM, Bahar G, Shpitzer T, Feinmesser R. Concomitant analysis of salivary tumor markers—a new diagnostic tool for oral cancer. *Clin Cancer Res*. 2006;12:3979–3984.