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Effects of smoking and gingival inflammation on salivary antioxidant capacity

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Abstract

Aim: This study evaluated possible effects of smoking and gingival inflammation on salivary antioxidants in gingivitis patients.

Methods: Twenty otherwise healthy gingivitis patients (10 self-reported smokers) and 20 periodontally and systemically healthy volunteer subjects were enrolled in the study. Whole saliva samples and full-mouth clinical periodontal recordings were obtained at baseline and one month following initial phase of treatment in gingivitis patients. Salivary cotinine, glutathione and ascorbic acid concentrations, and total antioxidant capacity were determined, and the data generated were tested by non-parametric tests.

Results: Salivary cotinine measurements resulted in re-classification of three self-reported non-smokers as smokers. Smoker patients revealed significantly higher probing depths but lower bleeding values than non-smoker patients ($p = 0.044$ and 0.001, respectively). Significant reductions in clinical recordings were obtained in non-smoker (all $p < 0.05$) and smoker (all $p < 0.01$) patients following periodontal treatment. Salivary total glutathione concentrations were reduced following therapy in gingivitis patients who smoke ($p < 0.01$). Otherwise, no statistically significant differences were found between the groups in biochemical parameters at baseline or following treatment ($p > 0.05$).

Conclusions: Within the limits of this study, neither smoking nor gingival inflammation compromised the antioxidant capacity of saliva in systemically healthy gingivitis patients.

Key words: ascorbic acid; cotinine; gingivitis/treatment; glutathione; saliva/total antioxidant capacity; smoking

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Smokers are more susceptible to advanced and aggressive forms of periodontal disease than non-smokers (Haber et al. 1993, Calsina et al. 2002). Additionally, smokers tend to respond less favourably to periodontal treatment (Ah et al. 1994, Renvert et al. 1998). However, the exact mechanisms by which smoking exerts detrimental effects on the periodontal tissues remain unclear. Tobacco smoking certainly modifies the periodontal response to microbial challenge (Barbour et al. 1997, Palmer et al. 2005). For example, smoking influences angiogenesis (Rezavandi et al. 2002, Cooke & Bitterman 2004), adhesion molecule profiles and leucocyte recruitment (Rezavandi et al. 2002, Scott & Palmer 2002), and multiple aspects of leucocyte development and function (Seow et al. 1994, Barbour et al. 1997, van Eeden & Hogg 2000, Palmer et al. 2005).

Reactive oxygen species play an important role in cell signalling and metabolic processes, but also contribute to pathogenic processes in a variety of inflammatory disorders (McCord 2000). Oxidative stress is defined as the disturbance of the pro-oxidant–antioxidant balance in favour of the former (Sies 1997). Unbalanced radical and non-radical reactive oxygen species can damage cells by variant mechanisms, including peroxidation of lipid membranes, protein inactivation, and induction of DNA damage, in addition to stimulating specific signalling pathways that lead to cytokine-induced tissue damage (Chapple 1997). It is well known that cigarette smoke contains a large amount of oxidative species, and therefore smoking represents a significant source of oxidative stress (Pryor et al. 1990, Pryor 1997, Palmer et al. 2005). In addition to this direct pro-oxidant burden, tobacco smoke can contribute to reactive oxygen species-mediated tissue damage through depletion of systemic endogen-

ous antioxidant capacity. For example, exposure of plasma to cigarette smoke in vitro causes depletion of vitamin C, ubiniquol-10, α-tocopherol, cryptoxanthin, retinol, and β-carotene (Eiserich et al. 1995, Lykkesfeldt et al. 1997). It is also known that, in vivo, smokers have lower serum vitamin C (ascorbic acid) and β-carotene levels, compared with non-smokers (Pryor et al. 1990, Tribble et al. 1993, Lykkesfeldt et al. 1997, Pryor 1997, Cowan et al. 1999). The reduced circulating levels of vitamin C noted in smokers have been hypothesized to be because of the activation of leucocytes, and subsequent generation of reactive oxygen species (Lykkesfeldt et al. 1997). Furthermore, large amounts of pro-oxidants are produced in prolonged inflammatory responses, as in gingivitis and periodontitis (Wei et al. 2004, Palmer et al. 2005). Therefore, both tobacco smoke and inflammation are sources of reactive oxygen species and can compromise the antioxidant capacity of serum and tissues.

Saliva possesses a variety of defence mechanisms responsible for the protection of the oral cavity from oxidative attacks, including uric acid, vitamin C, glutathione, and others (Halliwell 1991). Together with uric acid and albumin, ascorbic acid is among the major antioxidants in saliva (Diab-Ladki et al. 2003). However, as antioxidants work in concert, total antioxidant capacity is the most relevant parameter (Brock et al. 2004).

As yet, the relationship between smoking, gingival inflammation, and salivary antioxidant status has not been clarified. Possible alterations in the salivary antioxidant composition may influence clinical periodontal status as well as the response to mechanical periodontal therapy in smokers. Thus, the aims of this study were twofold: (1) to evaluate the possible effects of smoking and gingival inflammation on concentrations of total glutathione, ascorbic acid and on total antioxidant capacity in saliva; and (2) to investigate the possible effects of the initial phase of periodontal treatment on these parameters in otherwise healthy gingivitis patients.

Material and Methods

Study population

Twenty otherwise healthy chronic gingivitis patients (10 males, 10 females, with an age range of 25–58 years; 10 self-reported smokers) and 20 systemically and periodontally healthy volunteer subjects (10 males, 10 females, with an age range of 25–47 years; 10 self-reported smokers) were consecutively recruited. Diagnosis of plaque-induced gingivitis was made according to the criteria defined in the International Workshop for the Classification of Periodontal Diseases and Conditions (Mariotti 1999). Written, informed consent was obtained from all subjects. Exclusion criteria were systemic disease, medications that may influence the study, antibiotic or periodontal treatment in the previous 6 months, the use of mouthwashes, the regular intake of supplementary vitamins, and a past, but not current, smoking history (former smokers).

Initially, those who claimed to have never smoked were recruited into the non-smoker groups, while subjects who reported smoking $\geqslant 10$ cigarettes/day for more than 5 years were recruited into the smoker groups, and later reclassified according to salivary cotinine concentration. Subjects who smoked $\geqslant 10$ cigarettes/day for less than 5 years, and those who smoked < 10 cigarettes/day for more than 5 years were excluded in an attempt to make a clear discrimination between smokers and non-smokers. Subjects with gingivitis were recruited from those referred for treatment to the Dental School at Ege University, İzmir, Turkey. Healthy volunteer subjects with no history of periodontal disease (i.e., probing depths < 3 mm and no attachment loss, obvious clinical inflammation, or radiographic evidence of bone loss) were drawn from students and staff of Ege University.

Thus, subjects were initially placed into one of the four groups as follows:

Group 1: 10 healthy non-smoker subjects.
Group 2: 10 healthy smoker subjects.
Group 3: 10 non-smoker gingivitis subjects.
Group 4: 10 smoker gingivitis subjects.

Saliva sampling

Saliva samples were obtained in the morning following an overnight fast during which subjects were requested not to drink (except water) or chew gum. Whole saliva samples were obtained by expectorating into polypropylene tubes before clinical measurements at baseline and one month following initial periodontal treatment (scaling, root planing, and appropriate oral hygiene instructions). The saliva samples were first weighed and then immediately frozen at -40°C until the sample collection period was completed. The samples were then lyophilized and stored at -20°C until subsequent biochemical analyses.

Clinical measurements

Subsequent to saliva sampling, clinical periodontal recordings, including dichotomous plaque index (+/−), probing depth (PD), and presence of bleeding on probing (BoP; +/−), were performed at six sites on each tooth present, except the third molars, using a Williams probe. All clinical examinations were carried out by a single examiner (H. I.) who was blinded to the smoking status of the subject. Radiographic examination was also carried out to detect alveolar bone destruction in order to confirm diagnoses of gingivitis.

Laboratory Analyses

Measurement of salivary cotinine concentration

Salivary cotinine concentrations were determined by gas chromatography with nitrogen–phosphorus detection (Jacob et al. 1991a). The methodology had been modified for simultaneous extraction of nicotine and cotinine and determination using capillary gas liquid chromatography (GLC) (Jacob et al. 1991b). The internal standards, 5-methylnicotine and 1-methyl-5-(2-pyridyl)-pyrrolidin-2-one (''ortho-cotinine''), were provided by Peyton Jacob, III, Ph.D., Division of Clinical Pharmacology of the Department of Medicine, University of California, San Francisco.

Measurement of total antioxidant activity

The total antioxidant capacity of saliva samples was measured using an EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer's instructions. The total antioxidant capacity of saliva was determined by the ability of salivary antioxidants to inhibit the metmyoglobin-dependent oxidation of 2,2-azino-di-[3-ethylbenzthiazoline sulphonate] and related to the activity of a water-soluble vitamin E analogue (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; Trolox™, Cayman Chemical Company, Ann Arbor, MI, USA). Thus, total antioxidant capacity

is expressed in Trolox™ equivalents (μM). Microplates were read at 750 nm using a Victor^3 1420 multilabel counter (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA).

Determination of total glutathione content

Total salivary glutathione content was determined by using a kinetic enzymatic recycling assay, according to the manufacturer's instructions (Oxford Biomedical Research, Oxford, MI, USA). Reaction kinetics was measured by determining the rate of colour change at 405 nm using a Victor^3 1420 multilabel counter. The concentration of glutathione was expressed as micromolars of GSH (γ-glutamylcysteinylglycine) equivalents.

Determination of ascorbic acid content

Salivary ascorbate concentrations were determined, essentially, as previously described by Ihara et al. (2000). Briefly, proteins were first precipitated out using 4% metaphosphoric acid (final concentration). Ascorbic acid in saliva was subsequently oxidized by the nitroxide-free radical 2,2,5,5-tetramethyl-4-piperidin-1-oxyl (TEMPO) to dehydroascorbic acid, which, in turn, condenses with *o*-phenylenediamine to form a quinoxaline derivative that absorbs light at 340 nm (Ihara et al. 2000). Ascorbate concentrations were determined by extrapolating from standard curves generated by the same methodology using the principle that change in absorbance at 340 nm is proportional to ascorbate content. Absorbances were read on a Victor^3 1420 multilabel counter.

Statistical analysis

The Kruskall–Wallis test followed by the Mann–Whitney *U*-test was used for the group comparisons of the salivary antioxidant levels as well as the clinical periodontal measurements. Wilcoxon's signed-rank test was used to test the differences between baseline and post-therapy data within the same study group. Pearson's correlations were utilised to look at the relationships between salivary antioxidant levels and the clinical parameters.

Results

Clinical analyses

Demographic variables and mean values of clinical measurements are outlined in Table 1. As expected, PD, plaque index (PI), and BoP were higher in the gingivitis groups (Groups 3 and 4) when compared with the periodontally healthy groups (Groups 1 and 2, respectively) (all $p<0.01$). Smokers with gingivitis (Group 4) exhibited higher mean PD but lower mean PI) and BoP values than non-smokers with gingivitis (Group 3), all $p<0.05$. Smoker healthy subjects (Group 2) showed a significantly lower percentage of sites with BoP than healthy non-smokers (Group 1; $p<0.05$).

Significant reductions in PD, BoP, and PI were obtained in both non-smoker (all $p=0.01$) and smoker (all $p<0.05$) patients with gingivitis following periodontal treatment.

Biochemical analyses

Smoking status of subjects

Quantification of recent smoke exposure was made by the measurement of sali-

Table 1. Clinical characteristics of the study groups

	Group 1: non-smoker healthy	Group 2: smoker healthy	Group 3: non-smoker gingivitis	Group 4: smoker gingivitis	Non-smoker gingivitis (post-therapy)	Smoker gingivitis (post-therapy)
n	8	12	9	11	8	10
Male:female	5:3	5:7	4:5	6:5	4:4	5:5
Age	25.6 (1.1)	28.1 (4.4)	34.4 (10.5)	34.8 (10.2)	32. 8 (10.1)	34. 1 (10.4)
Number of teeth present	27.6 (0.7)	27.4 (1.4)	26.3 (3.1)	24.7 (4.0)	26. 4 (3.3)	25.5 (3.3)
Probing depth (mm)	0.84 (0.12)	0.86 (0.16)	1.19 (0.22)	1.38 (0.24)*	0.86 (1.55)†	0.91 (0.14)‡
Bleeding on probing (%)	9.0 (2.4)	5.0 (1.0)§	60.1 (5.1)	38.3 (12.3)*	12.1 (4.1)†	9.4 (3.5)‡
Plaque index (%)	11.0 (3.0)	13.2 (3.4)	68.0 (11.7)	61.8 (7.6)	14.8 (3.4)†	14.3 (5.6)‡

All data presented are mean (standard deviation) values.
*Significantly different than the non-smoker gingivitis patients (Group 3); $p<0.05$.
†Significantly lower than the baseline values of non-smoker gingivitis patients (Group 3); $p<0.05$.
‡Significantly lower than the baseline values of smoker gingivitis patients (Group 4); $p<0.01$.
§Significantly lower than the non-smoker controls (Group 1); $p<0.05$.

Table 2. Salivary cotinine, ascorbic acid, total glutathione concentrations and total antioxidant activity levels

	Group 1: non-smoker healthy	Group 2: smoker healthy	Group 3: non-smoker gingivitis (baseline)	Group 4: smoker gingivitis (baseline)	Non-smoker gingivitis (post-therapy)	Smoker gingivitis (post-therapy)
Wet weight (g)	4.7 (1.0)	4.7 (1.3)	4.9 (0.8)	4.7 (1.1)	4.4 (1.3)	4.5 (1.1)
Glutathione (μM)	3.3 (1.8)	3.8 (1.7)	4.6 (1.6)	4.7 (0.5)	4.0 (1.4)	1.0 (1.2)†
Ascorbic acid (mg/l)	1.88 (3.7)	0.8 (0.5)	1.8 (1.6)	1.6 (1.6)	1.2 (1.2)	1.2 (0.6)
TAC (mM)	0.14 (0.15)	0.24 (0.31)	0.24 (0.15)	0.13 (0.13)	0.25 (0.12)	0.10 (0.11)
Cotinine (ng/l)	1.9 (3.6)*	225.9 (134.6)	1.4 (1.9)*	152.3 (78.5)		

All data presented are mean (standard deviation) values.
*Significantly lower than the comparable smoking group; $p<0.001$.
†Significantly lower than all the other groups; $p<0.01$.

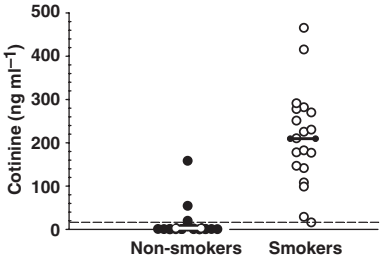

Fig. 1. Salivary cotinine concentrations in self-reported smokers ($n = 20$) and self-reported non-smokers ($n = 20$). Bars represent median values. The dotted line represents the cut-off concentration of 15 ng/ml pre-determined to differentiate biochemically defined smokers and non-smokers. Three self-reported non-smokers exhibited elevated salivary cotinine levels: thus these individuals were defined as smokers

vary cotinine concentrations, as presented in Table 2. Three self-reported non-smokers, who exhibited cotinine levels of >15 ng/ml (see Fig. 1), were re-assigned to the smoking group. Therefore, nine non-smoker (four males, five females), 11 smoker (six males, five females) gingivitis patients and eight non-smoker (five males, three females), 12 smoker (five males, seven females) healthy control subjects made up the study groups. In the re-assigned smoking groups, salivary cotinine levels ranged from 16.1 to 465.5 ng/ml, with the re-assigned non-smokers exhibiting salivary cotinine concentrations ranging from 0.1 to 2.3 ng/ml.

Salivary antioxidant measurements

Salivary antioxidant data at baseline and post-therapy, in the smokers and non-smokers, with and without gingivitis, are presented in Table 2. Mean salivary total glutathione concentrations were reduced post-therapy compared with baseline only in gingivitis patients who smoke (4.07 *versus* 1.0 mM, respectively; $p < 0.01$). However, no other statistically significant differences in any of the antioxidant indices were found between any of the groups, either at baseline or following treatment (all $p > 0.05$). Furthermore, Pearson's correlation analysis failed to reveal any statistically significant correlations between the clinical periodontal parameters and the salivary antioxidant concentrations ($p > 0.05$) (data not shown).

Discussion

Abundant cross-sectional data support the adverse relationship between smoking and periodontal diseases. Comprehensive reviews on this subject have been recently published by us and others (Barbour et al. 1997, Kinane & Chestnutt 2000, Johnson & Hill 2004, Palmer et al. 2005). A strong dose–response relationship between the amount smoked and the severity of periodontal destruction has also been shown, further supporting the role of smoking as a risk factor for periodontitis (Grossi et al. 1994, 1995, Calsina et al. 2002). Smokers are almost four times more likely to have severe periodontitis than non-smokers (Haber et al. 1993). However, the exact mechanisms by which smoking exerts its deleterious effects on periodontium remain unclear. One potential mechanism is through tissue damage mediated by oxidative species originating from tobacco smoke and tobacco-induced inflammation, in addition to the direct cigarette smoke-mediated depletion of antioxidants. To our knowledge, this is the first study to investigate the possible effects of both smoking and the initial phase of periodontal treatment on salivary antioxidant capacity in gingivitis patients.

Several methods of saliva collection are available, including the collection of unstimulated whole saliva; whole saliva stimulated with, typically, paraffin wax, gum base or citric acid; or the collection of saliva from specific salivary glands. For the purpose of analysing salivary antioxidant status, whole saliva is the most relevant, as it contains gingival crevicular fluid, immune cells, and tissue metabolites (Navazesh 1993, Kaufman & Lamster 2000) and reflects most closely the predominant intra-oral condition (Edgar 1992). Stimulation, on the other hand, may increase the flow of gingival crevicular fluid and this may result in false increases in the concentration of antioxidants in the saliva (Chapple et al. 1997). Accordingly, while we collected stimulated (i.e., expectorated) whole saliva, the degree of stimulation was minimal relative to that obtained when using gum, citric acid, or paraffin wax.

Self-reports of smoking status were inaccurately provided by three subjects, confirming the need to biochemically validate tobacco smoke exposures particularly in studies with small subject numbers, as we have previously described (Scott et al. 2001). After re-assigning these three subjects to the smoker groups, there was a very clear discrimination between smoker and non-smoker groups, reflected in significant differences in salivary cotinine concentrations, enabling us to evaluate the possible effects of smoking.

Antioxidants, by counteracting the harmful effects of free radicals, protect structural and tissue integrity. The antioxidant status of an individual is therefore of utmost importance (Zappacosta et al. 1999). Imbalances between levels of free radicals and reactive oxygen species and levels of antioxidants have been suggested to play an important role in the onset and development of several inflammatory oral diseases (Chapple 1997, Battino et al. 1999). Interestingly, Cowan et al. (1999) established that circulating concentrations of several antioxidants (α-tocopherol, retinol, lycopene or β-carotene) did not correlate with those in oral tissues, as had been previously implied. However, research on salivary antioxidants remains limited and conflicting data have been reported. Moore et al. (1994) have measured the antioxidant capacity of saliva in periodontally diseased and healthy individuals using the Trolox^{TM} equivalent assay and failed to find any significant difference between the groups. However, the disease status of the subjects was not defined clearly and clinical periodontal recordings in the controls were not reported in this early study. Chapple et al. (1997) found that the detection of differences in saliva total antioxidant capacity between periodontitis and control subjects depended upon whether salivary flow rates were accounted for in their data analysis. The same group (Brock et al. 2004) confirmed this in a study conducted with a well-defined study group of non-smokers, and found no differences in mean saliva total antioxidant capacity between groups, but did find a significantly higher total antioxidant delivery in unstimulated samples from health relative to periodontitis. There was also a gender bias, with males having higher saliva antioxidant capacity than females. Moreover, no significant correlations were found between the total antioxidant capacity of saliva and age or flow rate, regardless of periodontal status. Recently, the possible association of gingivitis and periodontitis with impaired salivary antioxidant status and increased oxidative injury has been further investigated by Sculley & Langley-Evans (2003). These researchers

reported that periodontal disease is associated with reduced salivary antioxidant status and increased oxidative damage within the oral cavity. However, periodontal health was assessed by CPITN index, which can be considered as rather rough for studies other than epidemiological ones. Furthermore, the authors concluded that evaluation of antioxidant capacity in gingival crevicular fluid is likely to be of greater importance for periodontitis than the more systemic changes in whole saliva.

Considering the wealth of in vitro and systemic in vivo data showing that tobacco smoke compromises antioxidant defences (Pryor et al. 1990, Tribble et al. 1993, Lykkesfeldt et al. 1997, Pryor 1997), it is critical to consider the influence of tobacco smoke on antioxidant status in the oral environment. The acute influence of smoking a single cigarette on concentrations of glutathione, uric acid, and total antioxidant activity measured in saliva has been addressed previously (Zappacosta et al. 1999). No statistically significant differences were found for uric acid or total antioxidant capacity before or after smoking or between smokers and non-smokers. However, salivary glutathione content was significantly higher in smokers and fell significantly after smoking one cigarette. The authors speculated that individuals with already low pre-smoking glutathione concentrations are most prone to the noxious effects of cigarette smoking. Recently, possible effects of smoking on total antioxidant capacity of total saliva and plasma samples have also been investigated by Charalabopoulos et al. (2005) in young, healthy males, who found no statistically significant differences in the salivary antioxidant defences of non-smokers and smokers, either before or after smoking.

The present study adds to the prior literature as it is the first to examine antioxidant status in gingivitis patients; and considers the confounding factor of inaccurate self-reporting of smoking status in medical and university environments, and also the influence of treatment in addition to cross-sectional baseline data. With regards to the cross-sectional data, our results concur with the previous studies, noted above, that have reported negative results. In the intervention arm of our study, total glutathione concentrations in saliva were reduced following successful therapy in gingivitis patients who smoke ($p<0.01$) but not in non-smokers. Ascorbate concentrations and total antioxidant capacities were unaffected by successful periodontal treatment (resulting in reduced plaque index, bleeding on probing, and probing depths), irrespective of smoking status.

In conclusion, therefore, in spite of the large inherent oxidative burden in inflammation and tobacco smoke, the results of this study suggest that — with the exception of glutathione in treated smokers with gingivitis — neither smoking or gingival inflammation, nor the combination of smoking and gingivitis is sufficient to significantly compromise salivary antioxidant defences in young adults with gingivitis. This does not preclude the possibility that tobacco-induced oxidant–antioxidant imbalances exist within the periodontal tissues themselves. Studies involving larger group sizes and analysis of GCF total antioxidant capacity are required to address these questions.

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Clinical Relevance

Scientific rationale for study: Tobacco smoke exposure and inflammation contribute to the oxidative burden of tissues. This study aimed to evaluate possible effects of smoking and gingival inflammation on salivary antioxidants and also of initial phase of periodontal treatment on salivary antioxidants in gingivitis patients.

Principal Findings: Other than a reduction in salivary total glutathione concentration following therapy in gingivitis patients who smoke, no significant differences were found in ascorbic acid concentrations and total antioxidant capacity between the groups at baseline or following treatment.

Practical Implications: Our findings suggest that neither smoking or gingival inflammation, nor combination of smoking and gingivitis is sufficient to compromise salivary antioxidant capacity in young adults with gingivitis.