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Effects of Cigarette Smoke on Salivary Protein Tyrosine Nitration

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Abstract
Introduction: Nitration of tyrosine and tyrosine-containing proteins and their roles in pathophysiology have just recently been reviewed. Despite low yields of tyrosine modifications, nitration of tyrosine residues may inactivate important proteins. Nitrotyrosine can be formed by various nitrating agents, including peroxynitrite. Thus, the occurrence of nitrotyrosine-containing proteins *in vivo* should be regarded as a general indication of tissue damage induced by reactive nitrogen species such as peroxynitrite. This strongly suggests that peroxynitrite could be formed *in vivo* under certain pathophysiological conditions.
Objective: Our aim in this study was to elucidate the effect of cigarette smoke (CS) on nitrotyrosine formation in saliva proteins.
Methods: We exposed saliva to CS, *in vitro*, and used Western Blotting (WB) and monoclonal anti-nitrotyrosine antibody to assess the level of saliva protein nitration.
Results: As saliva contains extensive amounts of nitrites, it was no surprise that at basal levels, saliva proteins, albumin, and α-amylase all were already nitrated. The WB also revealed that with continuous exposure to CS the tyrosine nitration of both albumin and α-amylase is declining significantly after 3 h. A quite similar effect was seen after exposure to aldehydes, but to a less extent as compared to CS. Exposure of nitrotyrosine-modified bovine serum albumin (BSA-N) to aldehydes, produced a similar effect, meaning a decrease in tyrosine nitration.
Conclusions: These findings might be explained by the possible ability of CS aldehydes to reduce protein-bound nitro group to an amine. Another proposed mechanism is that CS unsaturated aldehydes react with proteins mainly through Michael addition reaction; leading to the generation of stable aldehyde-protein adducts (APA). Thus, it may react with nitro groups of saliva proteins, like albumin or α-amylase, to generate APA, which ultimately, may not be recognized by our antibody. Another possible mechanism, is interaction between the aldehyde group with the hydroxyl group of the 3-nitrotyrosine, forming a hemiacetal, which is not recognized by the antibody. This mechanism might explain the difference in the 'denitration' effects caused by the saturated aldehyde acetaldehyde, which exists in large amounts in CS, and unsaturated aldehydes. Therefore, it is possible that the main player in the CS smoke "denitration" effect on salivary proteins is the aldehyde group and not the double bond of unsaturated aldehydes.

Key words: Tyrosine nitration, cigarette smoke, aldehydes, amylase

Introduction

Nowadays, about one third of adults in the USA are known to be smokers and smoking rates are increasing, especially among the female population [1, 2]. Cigarette smoke (CS) is presently known as the main cause of cancer, chronic bronchitis, emphysema, cardiovascular disease, and a leading cause of death worldwide [3]. Cigarette smoke is also a multipotent carcinogenic mixture that can cause cancer in numerous different organs, and a strong independent risk factor for cancers of oral cavity, upper respiratory tract, lung cancer and upper gastrointestinal tract [3-5]. CS contains over 4800 different chemicals, 400 of which are proven carcinogens. These carcinogens include aromatic amines, nitrosamines, oxidants such as oxygen free radicals and also high concentrations of toxic volatile aldehydes. All are, presumably, major causes of damage to various biomolecules exposed to CS [6].

It is well established that CS alters saliva components leading to a decrease in the ability of saliva to act as a protective fluid against oxidative damage, carcinogens, bacterial overgrowth and more. The mechanisms by which CS alters saliva components and activity is not fully understood. In the human oral cavity, nitrate secreted as a salivary component is reduced to nitrite and nitric oxide (NO) by certain bacteria, and salivary nitrite may be transformed to NO, NO_2, and N_2O_3 which can lead to tyrosine nitration. It is well known that NO is an antimicrobial compound as well as a physiologically important compound. The concentration of nitrate in saliva (0.2–2.5 mM) is depen-

dent on the amount of nitrate ingested. The concentration of nitrite in saliva (0.05–1.0 mM) is dependent on the concentration of nitrate. The nitrite and NO formed in the human oral cavity can be oxidized by molecular oxygen and by salivary peroxidase producing NO_2^- [7].

The concomitant production of NO and superoxide constitutes the main source of tyrosine nitration. Known sites of tyrosine nitration appear to be in close proximity to acidic residues, most containing turn inducing residues, but not cysteine or methionine residues. In most reported studies, nitration of tyrosine has been associated with a significant loss of function of the nitrated protein. Protein nitration has been utilized as a biological marker to monitor disease onset, progression, and outcome. A number of studies have also indicated that nitration of proteins at least *in vitro* and in cell model systems could significantly alter protein function, alter protein turnover, influence immune responses, and possibly be involved in signal transduction processes.

In addition to the potential of protein nitration to hinder protein function, recent data raise the issue of whether protein nitration might also be a cellular signaling mechanism. To be considered a cellular signaling mechanism, protein nitration must meet four basic criteria: controlled rates of formation, specificity, modification of target protein and cell function, and reversibility. The specificity of protein nitration and modification of protein and cell functions by protein nitration have been demonstrated. However, very little evidence exists regarding the reversibility by a protein "denitration" process *in vivo*. Recently Koeck et al [8] discovered that isolated rat liver mitochondria are capable of nitrotyrosine clearance during a 20-min episode of hypoxia-anoxia and that nitrotyrosine immunoreactivity reappears during reoxygenation in an L-arginine-dependent way at protein spots that matched the spots before hypoxia- anoxia [8]. However, there is no mentioning in the literature of 'nitratase' or denitrating enzymes in human saliva or plasma.

Many of the abnormalities associated with CS, including endothelial dysfunction, proinflammatory effects on vessel wall, prothrombotic effects such as increased platelet reactivity, reduced endogenous fibrinolysis and lipid peroxidation can largely be explained by the effect of increased oxidative and nitrosative stress [7]. Our working hypothesis was that when CS contacts saliva, there is a massive increase in reactive oxygen species (ROS), reactive nitrogen species (RNS), and aldehydes which, in turn, cause protein alterations in the form of tyrosine nitration. As a result, there is a decline in the defense mechanism of the saliva especially in its antioxidative properties. Thus, external addition of antioxidants can neutralize the effect of CS-originated oxidants and could act as an effective means in neutralizing the toxic effect of smoking.

Material and Methods

The study was performed in accord with the Declaration of Helsinki for Human Research and the study protocol was approved by an institutional Ethics Committee.

Whole saliva, which is basically total oral fluid, was collected from healthy male and female nonsmokers under non-stimulatory conditions in the morning. For the collection, which was performed at least 1 h after eating; volunteers were asked to generate saliva in their mouths and to spit it into a wide test tube for 10-15 min. Following collection, saliva was immediately centrifuged (1000 x *g*, 3 min) to remove squamous cells and cell debris. The fresh supernatant was immediately used for CS and aldehydes studies, and tyrosine nitration studies were performed.

The cigarettes used in this study were popular commercial cigarettes ('Time' cigarettes; Dubek Ltd., Tel Aviv, Israel) containing 14 mg of tar and 0.9 mg of nicotine per cigarette. Acrolein and crotonaldehyde were purchased from Sigma-Aldrich (St. Louis, MO), acetaldehyde from Fisher Scientific

Exposure of Saliva to Cigarette Smoke (CS)

An *in vitro* study was carried out using 'Time' cigarettes combined with a vacuum system, as described previously [6, 9]. Saliva (4–5 ml) was placed in 250 ml flasks with a sidearm to which the cigarettes were attached. A reproducible vacuum was created in the flask, and after opening the vacuum to the lighted cigarette for 5 s, 80 to 100 ml of CS 'puffs' were drawn into the flask. Flasks were incubated in a metabolic shaker for a total of 3 h at 37 °C. In a given experiment, puffs were administered to the flask over a 3-h time period at 20-min intervals, for a total of nine times. Samples for biochemical analysis were removed from the flasks at zero time and then at 1, 2, and 3 h.

Exposure of Saliva to Purified Aldehydes

4-5 ml of human saliva was treated with an aldehyde mixture of crotonaldehyde and acrolein (unsaturated aldehydes) similar to that reported to be present in a mainstream smoke of a single 2R1 University of Kentucky reference cigarette (5), to give a final concentration of 0.8 μmol/4 ml acrolein and 0.21 μmol/4 ml crotonaldehyde (AL). Another 4-5 ml of saliva was treated with acetaldehyde (saturated aldehydes) (ACET-AL) to give a final concentration of 20 μmol/4 ml. Aldehydes were administered to the flask over a 3-h time period at 20-min intervals, for a total of nine times. Samples for biochemical analysis were removed from the flasks at zero time and at 1, 2, and 3 h. Flasks were incubated in a metabolic shaker for a total of 3 h at 37 °C.

For positive control, we exposed bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and nitrotyrosine-modified BSA (BSA-N) (Alexis Corporation, Lausen, Switzerland) at parallel amounts to those of saliva albumin to volatile aldehydes in the same manner.

Addition of Glutathione (GSH) to Saliva

GSH was added to saliva at zero time and flasks were incubated in a metabolic shaker for a total of 30 min at 37 °C, prior to the first smoking puff or aldehyde addition, at concentrations of 1mM (GSH from Sig-

ma-Aldrich, St. Louis, MO). In addition, in all experiments, a control without the compounds was run with no difference in the assay conditions.

Nitrite Concentrations Measurement - Griess Reagent Assay

As a measure of the intensity of CS puffs, the nitrite levels in saliva were assessed using the commercially available Griess reagent [10] (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. The products of the reaction between nitrites and Griess reagent were measured spectrophotometrically at 540 nm and calibrated against the calibration curve constructed with the use of $NaNO_2$.

Protein Immunoblotting (Western Blot)

Following the various treatments, the saliva samples were spinned down for 1.5 min, boiled for 10 min at 95 °C in sample buffer and separated on 10% Acrylamide SDS-PAGE. Monoclonal antibody to nitrotyrosine for nitration detection (Chemicon International, Billerica, MA, USA), Secondary antibody was goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to manufacturer's instructions. Densitometric quantitative analysis of the protein bands detected by Western blot, were carried out using "Tina" software (ISBE University of Manchester, UK).

Statistical Analysis

Statistical analysis was performed using an unpaired t-test. To determine statistical significance, the ranges, means, and SD were computed. Results are reported as means ±SD. Statistical significance was set at $P<0.05$.

Results

The Effect of CS and Volatile Aldehydes on Salivary Protein Tyrosine Nitration

We checked whether exposure of human saliva to CS and aldehydes is associated with more nitration of tyrosine residues of saliva proteins. We exposed the saliva proteins to immunoblotting by PAGE using antinitrotyrosine antibody (in all the results n = 3).

As shown in Fig. 1A, saliva proteins (albumin and α-amylase) were both already nitrated at time 0 and exposure to CS for 3 h caused a decrease in tyrosine nitration of both albumin and α-amylase (65 ±6.3% and 80 ±7.8%, respectively, $P<0.001$ in comparison to time 0). As we received these results we examined if there is a change in protein concentration by the Bio-Rad protein assay. There was no reduction in protein during exposure to CS for 3h (data not shown). Fig. 1B shows that exposure to air did not induce any change in saliva protein tyrosine nitration during 3 h.

As a mean to understand our results we decided to expose BSA to CS. BSA did not show any tyrosine nitration at time zero and after 3 h (data not shown). As BSA-N comes in very small amounts, it was impossible to expose it to CS. It is also important to mention

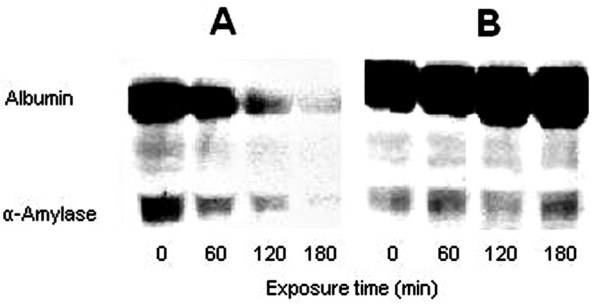

Fig. 1. Immunoblotting of antinitrotyrosine Ab using PAGE analysis of salivary proteins at zero time and after 3-h exposure to cigarette smoke - CS (A) and air (B).

that we examined saliva albumin and not serum albumin; therefore they probably behave in a different manner. Exposure of purified human saliva α-amylase to CS did not produce any change in tyrosine nitration, and it was not nitrated from the beginning like α-amylase in our saliva (data not shown). We continued with our research and determined the possible damage of CS to saliva proteins and enzymes by adding aldehydes and examined their effect on tyrosine nitration.

Effects of Unsaturated and Saturated Aldehydes on Salivary Protein Tyrosine Nitration

As shown in Fig. 2A, unsaturated aldehydes, acrolein and crotonaldehyde, induced a similar to CS effects and reduced tyrosine nitration of albumin and α-amylase after 3 h (20 ±1.8% and 40 ±3.4%, respectively), but to a much less extent in comparison to CS ($P<0.001$). The saturated aldehyde, acetaldehyde, had the most powerful effect in reducing tyrosine nitration of albumin after 3 h in comparison to both CS and unsaturated aldehyde (96 ±12% *vs.* 80 ±7.8% *vs.* 40 ±3.4% respectively; $P<0.001$ compared to time 0) and caused complete "denitration" of α-amylase (Fig. 2B).

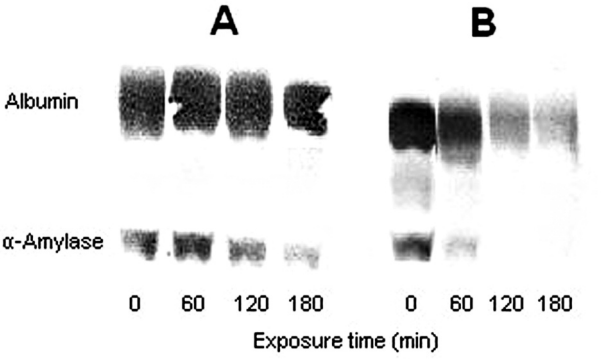

Fig. 2. Immunoblotting of antinitrotyrosine Ab using PAGE analysis of salivary proteins at zero time and after 3-h exposure to unsaturated (A) and saturated aldehydes (B).

To verify our results we exposed BSA and nitrated BSA (BSA-N) to unsaturated aldehydes and acetalde-

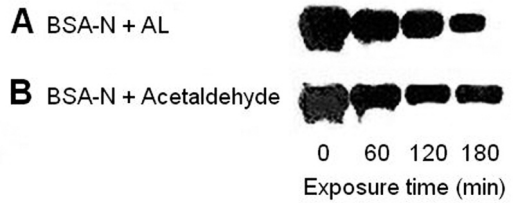

Fig. 3. Immunoblotting of antinitrotyrosine Ab using PAGE analysis of BSA-N at zero time and after 3-h exposure to unsaturated (A) and saturated aldehydes (B).

hyde. BSA produced no tyrosine nitration at all times (data not shown). On the other hand, exposure of BSA-N to aldehydes produced a similar effect to that of human saliva albumin, meaning, there was "denitration" of tyrosine after 3 h (Fig. 3A and B). Exposure of purified human saliva α-amylase to aldehydes did not produce any change in tyrosine nitration, and it was not nitrated from the beginning like α-amylase in our saliva (data not shown).

Effects of GSH on Tyrosine Nitration of Saliva Proteins

To elucidate whether GSH had an effect on this phenomenon, and as described before, we incubated the saliva with GSH prior to exposure to CS and aldehydes. We found that there was some protective effect of GSH against the decrease in tyrosine nitration.

Effects of CS on Salivary Protein Tyrosine Nitration in the Presence of GSH

Fig. 4 shows the effect of 1mM GSH addition to saliva and exposure to CS. It is noticed that GSH had a significant effect against CS denitration of albumin (20 ±4.2% *vs.* 65 ±6.3%, P<0.001) and α-amylase denitration (46 ±3.8% *vs.* 80 ±7.8%, P< 0.05).

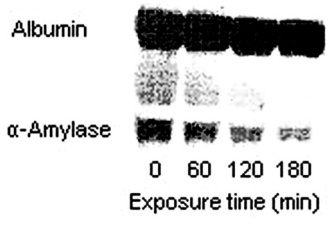

Fig. 4. Immunoblotting of antinitrotyrosine Ab using PAGE analysis of salivary proteins at zero time and after 3-h exposure to CS+GSH.

Effects of Unsaturated and Saturated Aldehydes on Salivary Protein Tyrosine Nitration in the Presence of GSH

Fig. 5A shows the effect of 1 mM GSH addition to saliva and exposure to unsaturated aldehydes. GSH had a significant effect against unsaturated aldehydes denitration of both saliva albumin (3 ±0.7% *vs.* 20 ±1.8 %, P<0.001) and α-amylase (10 ±1.7% *vs.* 40 ±3.4%, P< 0.001). Fig. 5B shows the effect of 1 mM GSH addition to saliva and exposure to saturated aldehyde, acetaldehyde. GSH had a significant effect against acetaldehyde denitration of both saliva albumin (19 ±3.1% *vs.* 48 ±5.9%, P<0.001) and α-amylase (52 ±4.9% *vs.* 96 ±12%, P<0.001).

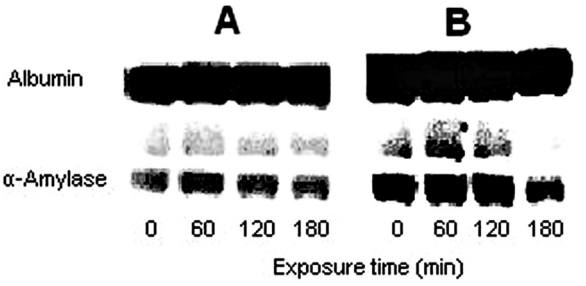

Fig. 5. Immunoblotting of antinitrotyrosine Ab using PAGE analysis of salivary proteins at zero time and after 3-h exposure to unsaturated aldehydes + GSH (A) and saturated aldehyde (B).

Discussion

Cigarette smoke is associated with a variety of human pathologies including atherosclerosis, CVD and cancer [11]. Ma et al [12] recently demonstrated that oxidative and nitrative stress contributes to the development of oral carcinogenesis through DNA damage. RNS in the form NO_3 and NO_2 and aldehydes, 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. Salivary nitrosamine production and metabolism are also based on the dietary 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 and thiocyanate [13].

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 [14, 15]. Salivary composition of OSCC patients is substantially altered with respect to free radical-involved mechanisms. All salivary RNS analyzed in these patients 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 [7].

Several human studies have investigated the effects of smoking on salivary damage. However, the exact mechanism by which CS components cause this damage is not fully understood. Therefore, we have decided to study the effects of CS components, especially volatile aldehydes, on salivary damage through nitration of enzymes and proteins. Nitration of tyrosine

and tyrosine-containing proteins and their roles in pathophysiology have recently been reviewed by Ischiropoulos [16]. Despite low yields of tyrosine modifications, nitration of tyrosine residues inactivates important proteins [17-22].

Nitration of tyrosine residues by peroxynitrite disrupts the phosphorylation of tyrosine residues in proteins involved in cell signaling networks [23]. However, more than a decade ago, an enzymatic activity, which modifies nitrotyrosine containing proteins (a 'nitrotyrosine denitrase'), has been reported in homogenates of rat tissue. The activity is increased about twofold in spleen extracts after endotoxin (bacterial lipopolysaccharide) treatment of animals, suggesting that the activity is inducible or regulated [24]. Thus, nitration of tyrosine could be one of the important post-translational modifications of proteins, the levels of which should be regulated biologically. It should be noted that nitrotyrosine can be formed by various nitrating agents, including peroxynitrite, nitrous acid, N_2O_3 and NO_2 [25, 26]. NO may also react directly with tyrosyl radicals (e.g., stable radicals such as that found in ribonucleotide reductase or those formed by H_2O_2 or others oxidants) to form nitroso or nitro derivatives. Thus, the occurrence of nitrotyrosine-containing proteins *in vivo* should be regarded as a general indication of tissue damage induced by reactive nitrogen species such as peroxynitrite. As one of our aims was to elucidate the effect of CS on nitrotyrosine formation, we used WB and exposed our saliva proteins to monoclonal anti-nitrotyrosine antibody. As saliva contains extensive amounts of nitrites, it was no surprise that at basal levels, saliva proteins, albumin and α-amylase, were already nitrated.

The WB also revealed that with exposure to CS the tyrosine nitration of both albumin and α-amylase is declining significantly after 3h (65% and 80%, respectively in comparison to time 0 (Fig. 1). A quite similar effect was seen after exposure to unsaturated aldehydes, but to a less extent as compared to CS (Fig. 2A). A phenomenon that kept repeating itself was the significant effect of acetaldehyde on the "denitration" of albumin and especially α-amylase-48% for albumin and 96% for α-amylase (Fig. 2B). At this stage we decided to verify whether our results are real "denitration" from tyrosine residues or non specific findings. We exposed nitrotyrosine-modified BSA (BSA-N) to unsaturated aldehydes and acetaldehyde, and received a similar effect, meaning a decrease in tyrosine nitration (Fig. 3A and B). For technical reasons it was not possible to expose BSA-N to CS. Exposure of pure BSA and pure α-amylase to CS and volatile aldehydes produced no tyrosine nitration at all times (data not shown).

These findings might be explained by the possible ability of aldehydes to reduce protein-bound nitro group to an amine [27]. Thus, if aldehydes can reduce nitro group of proteins to an amine, it would result in the vanishing of nitrotyrosine modified proteins. Another proposed mechanism is that aldehydes react with proteins mainly through Michael addition reaction; leading to the generation of stable aldehyde-protein adducts (APA). Thus, it may react with nitro-groups of saliva proteins, like albumin or α-amylase, to generate APA, which, ultimately, may not be recognized by our antibody [28]. Another possible mechanism, is interaction between the aldehyde group with the hydroxyl group of the 3-nitrotyrosine, forming a hemiacetal, which is not recognized by the antibody (Fig. 6). This mechanism might explain the difference in "denitration" between saturated aldehyde, acetaldehyde, which exists in large amounts in CS, and unsaturated aldehydes. It is possible that the main player in "denitration" of salivary proteins is the aldehyde group and not the double bond of unsaturated aldehydes.

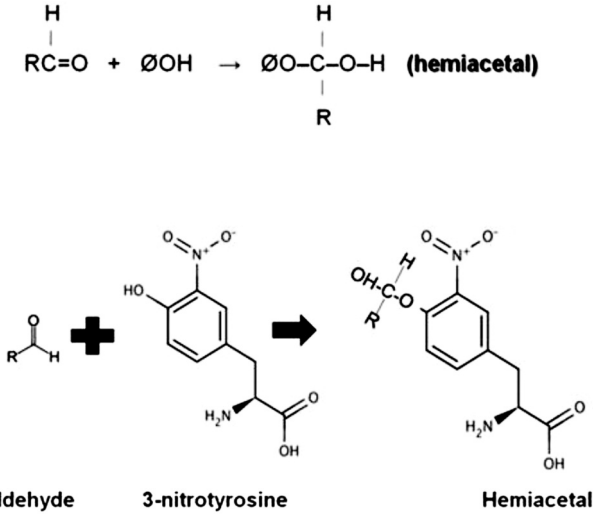

Fig. 6. Possible mechanism for aldehyde-induced 'denitration'.

These results were confirmed when we added GSH to saliva prior to exposure to CS and aldehydes and received a significant effect of protection against "denitration" of tyrosine. The effect was more intense for tyrosine nitration of albumin than α-amylase, but for all experiments there was more than 50% protection (Fig. 4-5). GSH is also able to interact with the aldehyde groups of different aldehydes.

Reviewing the literature, this is the first time we saw that there is tyrosine nitration at basal levels. This could be explained by the large presence of nitrates and nitrites in human saliva. They are increased due to the presence of microbial flora in the oral cavity and have a role as antimicrobial chemicals. Even though our aim was to show an increase in tyrosine nitration after exposure to CS, we received a quite interesting result, suggesting protein alterations which lead, in the case of α-amylase, to reduced activity.

Our results together with previous studies we have conducted [29, 30] suggest an explanation for the involvement of aldehydes and RNS in damage to salivary components and a possible mechanism of CS induced damage to saliva, which may lead to the progression of oral cavity associated diseases. Future *in vitro* studies should include using saliva from smokers and comparing to non-smokers and *in vivo* studies of both smokers and non-smokers and treatment with antioxidants supplements, such as GSH and epigallocatechine-3-gallate (EGCG) that directly reacts with

tyrosyl radicals, thus preventing their transformation into nitrotyrosine.

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Conflicts of interest: The authors declare no conflicts of interest in relation to this article.

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