Short communication

Effect of gas phase and particulate phase of cigarette smoke on salivary antioxidants. What can be the role of vitamin C and pyridoxine?

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Abstract:
The effect of smoking is in our days a serious global public health problem of major concern. Incidence of oral squamous cell carcinoma (SCC) in cigarette smokers is four to seven times higher than in nonsmokers. There is a constant and direct attack of various cigarette smoke constituents on the oral epithelial cells, which gradually accumulate and cause malignant transformation.

Saliva is the first biological fluid that encounters inhaled cigarette smoke (CS). We have studied the influence of CS on salivary antioxidant capacity, uric acid, amylase and LDH (lactate dehydrogenase). In our study both, gas and particulate phase of CS were tested separately, and possible antioxidant effect of pyridoxine on salivary components was examined.

Our results indicate that exposure to both, gas and particulate phase of CS caused a statistically significant decrease in salivary uric acid, LDH and amylase activity.

We have also studied the effect of vitamin C (10 mg/dl) and vitamin B₆ (1 mM) during incubation of saliva in the presence of CS. The addition of vitamin C had a significant (p < 0.05) protective effect on salivary uric acid level (0.25 ± 0.12 for saliva incubated with gas phase of CS vs. 0.65 ± 0.12 for saliva incubated with gas phase of CS in the presence of vitamin C). Vitamin C was not able to maintain/restore the original uric acid level. In the presence of the gas phase, pyridoxine had no protective effect, neither on salivary uric acid level nor on the FRAP activity of saliva.

The purpose of our study was to discover a connection between the level of antioxidants in saliva in the presence of the two components of CS. Our results show that salivary antioxidant system is significantly and distinctly affected by both gas and particulate phase of CS and suggest that an adequate intake of antioxidants may help smokers to avoid CS-induced oxidative damage and to prevent degenerative diseases.

Key words: cigarette smoke, oxidative stress, uric acid, salivary antioxidant system
Introduction

Oral squamous cell carcinoma (SCC) is the most common malignancy of the head and neck, with a worldwide incidence of over 300,000 new cases annually. It is a disease characterized by a high morbidity and mortality rate of about 50% [4, 10, 11, 13]. Nicotine, the major constituent of tobacco, is responsible for the compulsive use of tobacco and its role has been largely studied and reviewed [19–21]; however nicotine is only one of the thousands of compounds present in tobacco or that can be produced during combustion. On the other hand, one of the major inducers of SCC is exposure to cigarette smoke (CS), responsible for 50–90% of the cases [5, 8, 9]. CS, which contains several carcinogens, known to initiate and promote tumorigenesis and metastasis, is the major cause of oral cancer [12].

The two phases of cigarette combustion product can be distinguished: the gas phase and the solid particulate phase (also known as tar phase). Particulate is the material retained on a filter, whereas gas phase smoke passes through the filter. In fact, CS contains over 4,000 different chemicals (out of which 3,000 are in the gas phase and 1,000 in the particulate phase). Four hundred of them have been proven to be carcinogens [4]. Both the particulate and gas-phase smoke are very rich sources of free radicals [6, 17]. Ko et al. [8] showed that reactive oxygen species (ROS) do not exist in either unburned tobacco leaves or in cigarette ash. ROS in CS are created through combustion and exist in the gas phase or are attached to suspended particles in the particulate-phase.

Saliva is the first biological fluid that encounters the inhaled CS. The anticarcinogenic potential of saliva has been already demonstrated in a study where it was shown that saliva could significantly inhibit the initiation and progression of oral cancer in animal models [5]. The salivary antioxidant system plays a very important role in the anticarcinogenic capacity of saliva [18]. It includes various molecules, the most important of which are uric acid and the peroxidase system. Uric acid contributes to approximately 70% of the total salivary antioxidant capacity [15]. This antioxidant system can be significantly affected by free radicals from CS.

Some toxic components of CS, unsaturated and saturated aldehydes, could interact with thiol-rich compounds, leading to structural and functional modification of these molecules [23].

The aim of this study was to evaluate the relationship between the two phases of CS (gas-phase and particulate phase), and salivary uric acid concentration and the activity of two important salivary enzymes (amylose and lactate dehydrogenase – LDH) in producing oral oxidative stress, one of the most important steps to malignant transformations.

The balance between oxidants and antioxidants in blood and saliva can be influenced by antioxidant administration, but the specific mechanism is still unknown. Therefore, we have studied the possible protective effect of some antioxidants (vitamin C 10 mg/dl and B6 1 mM) during incubation of saliva with the components of CS.

Materials and Methods

Whole saliva was collected from 6 healthy nonsmokers (3 women and 3 men). Collection was always performed between 9 and 10 a.m. to avoid circadian variations. For the experiments we used combined saliva from the 6 volunteers [14].

The cigarettes used in this study were commercially available Marlboro cigarettes.

Exposure of saliva to CS was conducted as described earlier [16]. A commercial filter-tipped cigarette (80 mm) with particulate phase content of 6 mg, nicotine 0.5 mg and carbon monoxide 7 mg was mounted in a 500 μl pipette tip that penetrated the hole in the stopper of the flask containing 1 ml of saliva and extended down past the side arm connected to a water pump and ended about half way of the bottom of the flask. The cigarette was lit and puffs of CS were introduced into the flask till the cigarette was finished. Saliva was incubated 1 h in the presence of CS at 37°C.

After incubation saliva was immediately centrifuged at 800 x g for 10 min to remove squamous cells and cell debris. The resulting supernatant was analyzed. Before CS incubation vitamin C and vitamin B6 were added to 1 ml of saliva at a final concentration of 10 mg/dl vitamin C and 1 mM vitamin B6.

Uric acid determination was performed on a Hospitex EOS BRAVO (Italy) automatic analyzer using analyzing kits from Diasys, Germany.
All enzymatic analyses were performed on a Hopsitex EOS BRAVO automatic analyzer using analyzing kits from Diasys, Germany.

For the salivary antioxidant capacity, we have used the ferric reducing ability (FRAP) method, according to Vassalle et al. [22].

Results are presented as the means ± SD. Statistical comparisons were performed by Student’s t-test. A value of p < 0.05 was considered statistically significant.

Results and Discussion

It has been suggested that free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the inhaled cigarette smoke induce a gradually evolving process, initially expressed by dysplastic lesions of the mucosa, which are transformed into in situ carcinoma lesions and eventually result in full blown infiltrating and metastasizing oral SCC [12]. Further evidence for the suggested role of free radicals in the pathogenesis of evolving oral SCC is based on a recent study [8] demonstrating that ROS, such as hydroxyl radical, are formed in the human oral cavity during areca quid chewing, and that they might cause oxidative DNA damage in the surrounding tissues. In the light of these findings, the salivary anticarcinogenic capacity, which has only recently been recognized, may be attributable to its antioxidant system, which is mostly based on uric acid [2].

Figures 1 and 2 show the results obtained after saliva was incubated with gas phase of CS.

As shown in Figure 1, the exposure to the gas phase of CS caused a statistically significant decrease in salivary uric acid (p < 0.05).

Addition of 10 mg/dl of vitamin C had a significantly (p < 0.05) protective effect on salivary uric acid level (0.25 ± 0.12 for saliva incubated with gas phase of CS vs. 0.65 ± 0.12 for saliva incubated with gas phase of CS in the presence of vitamin C). Addition of vitamin C was not able to maintain/restore the original uric acid level but it was effective in ameliorating the condition found after gas-phase treatment.

In the presence of gas phase of CS, pyridoxine had no protective effect neither on salivary uric acid level nor on the FRAP activity of saliva.

FRAP assay involves [6, 22] neither an oxidant nor an oxidizable substrate but it measures the ability to reduce the ferric ions present in the assay mixture to ferrous ions. Even if the iron ions are given exogenously in the assay, in order to magnify the phenomenon, this test gives a valuable information about the reductants present in biological fluids. Importantly, as illustrated by our experimental data, in the presence of gas phase of CS, salivary FRAP activity significantly (p < 0.05) increased compared with unincubated saliva (Fig. 1). It must be borne in mind that not all reductants able to reduce ferric ions are actually...
antioxidants [3, 22]. This could explain our results. Gas phase of CS contains agents (which are not antioxidants) with great ability to reduce ferric ions and produce large amounts of ferrous ions. Ferrous ions react with hydrogen peroxide producing hydroxyl radicals and cause a powerful oral oxidative stress.

*In vitro* exposure of saliva to gas phase of CS for 1 h showed a significant (p < 0.05) decrease in salivary LDH activity (88.3%) and amylase activity (84.57%) (Fig. 2). Addition of 10 mg/dl of vitamin C did not protect salivary LDH and amylase activities during exposure to gas phase of CS (Fig. 2).

It has been shown [7] that pyridoxine could have antioxidant activities. However, in our study there was no significant effect of pyridoxine on salivary LDH and amylase activities exposed to gas phase of CS suggesting that vitamin B₆ is unable to protect affected sulphydryl groups important for catalytic activities of LDH and amylase.

The results concerning the situation of saliva after having been exposed to particulate-phase CS are reported in Figure 3 and Figure 4.

As shown in Figure 3, our results indicate that exposure to CS particulate phase caused a statistically significant decrease in salivary uric acid (by 98%; p < 0.05).

Addition of 10 mg/dl of vitamin C had a significant protective effect on salivary uric acid level (0.02 ± 0.005 mg/dl for incubated saliva with particulate-phase CS vs. 0.45 ± 0.11 mg/dl for incubated saliva with gas-phase CS in the presence of vitamin C).

In the presence of the particulate phase, pyridoxine had no protective effect, neither on salivary uric acid level nor on the FRAP activity of saliva.

Experimental data obtained in the presence of particulate phase of CS indicated that salivary FRAP activity significantly (p < 0.05) increased compared with unincubated saliva (Fig. 3).

Aqueous extracts of cigarette smoke particulate phase (ACT) contain a low-molecular-weight quinone-hydroquinone-semiquinone system (Q–QH₂–QH). Since ACT contain the quinone radical system, it can reduce oxygen to the superoxide form (equation 1).

\[ QH + O₂ → Q + O₂⁻ + H⁺ \]  

Superoxide, in turn, can be converted by superoxide dismutase, to form hydrogen peroxide (equation 2) [15].

\[ 2O₂⁻ + 2H⁺ → O₂ + H₂O₂ \]

Thus, the ACT solutions consume oxygen and produce a series of activated oxygen species that can cause biological damage.
Furthermore, both cellular fluids and cigarette smoke particulate phase themselves contain metal ions (such as iron) that can catalyze the production of hydroxyl radicals from hydrogen peroxide via the Fenton reaction (equation 3):

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}^+ + \text{HO}^- + \text{Fe}^{3+} \] (3)

Thus, the reducing capability of cigarette smoke particulate phase might explain our results obtained using the FRAP method. The reducing capability of cigarette smoke particulate phase causes the formation of superoxide, which ultimately leads to the production of the hydroxyl radical which is a very toxic oxidant.

In conclusion, in our study, the FRAP method can be regarded as an oxidant index rather then a method for measuring total antioxidant capacity.

In vitro exposure of saliva to CS particulate phase for 1 h showed a significant (p < 0.05) decrease in salivary LDH activity (82.27%) and amylase activity (40%) (Fig. 4).

Addition of 10 mg/dl of vitamin C caused further decrease in salivary LDH and amylase activities (probably by oxidation), during exposure to the particulate phase CS (Fig. 4). The presence of redox active metals (iron, copper) in saliva [5] has already been shown, whereas vitamin C in the presence of redox-active metals may act as pro-oxidant agent rather than an antioxidant [17] and such behavior could explain these experimental findings.

Pyridoxine had no protective effect on salivary LDH and amylase activities exposed to CS particulate phase.

Our results show that salivary antioxidant system is significantly and distinctly affected by the both CS gas and particulate phase. Salivary uric acid level is more affected by particulate phase of CS compared with gas phase, and vitamin C seems to be a more efficient antioxidant during gas phase exposure. Uric acid is an antioxidant that chelates metal ions from the particulate phase.

The loss of enzymatic activities presented in our study may be one of the important mechanisms by which CS initiates oral inflammatory diseases, pre- and malignant transformations.

The design of molecules which can inhibit the interaction between CS components and thiol or amino groups of salivary proteins and “scavenge” free radicals could be of great interest.

Since CS was found to inactivate salivary amylase and LDH, it is possible that other important protective salivary enzymes, such as salivary peroxidase system, may also have been affected. Further studies are needed to elucidate these inhibitory mechanisms.

Further investigations will be necessary in order to test the protective effect of pyridoxamine, if there is any, on the possible interactions of carbonyl compounds from gas or particulate phase of CS with amino groups of salivary proteins.

Over the last decade, oral cancer has represented 2–4% of malignant tumors and in the last 5 years the mortality rate has reached the level of approximately 50%, not to mention the esthetic and functional consequences affecting patients.

The increase in the survival rate of these patients depends on diagnosis, prognosis and treatment of incipient lesions and pre-malignant lesions, resulting in a decrease in consequences of oncologic therapy.

Changes in the antioxidant enzymes in saliva suggest that this biological fluid is suitable for the prognosis and evolution of oral degenerative diseases [1].

The purpose of our study was to discover a connection between the level of antioxidants in saliva in the presence of the two components of CS.

Our results suggest that vitamin C (under our specific experimental condition, at a final concentration of 10 mg/dl) may have a significant protective effect on salivary uric acid level, which is the most important oral antioxidant, while, on the contrary, pyridoxine had no protective effect. Even if these results should be confirmed in other conditions, we think that these results may be of great interest in the near future. Further studies with other antioxidants are also needed to ascertain if an adequate antioxidants intake could actually help smokers to avoid CS-induced oxidative damage in oral cavity and in this way to prevent some degenerative diseases.

Moreover, we hope to create a strategy based on these data capable of identifying the prognosis of a specific lesion before malignant transformation in smokers.

A large part of the world population continues to smoke ignoring the campaigns and warnings. Therefore, it is imperative to find a new prevention and treatment strategies against the complication of these oral diseases and the improvement of saliva antioxidant status may represent one of the possible solutions.
References:


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