Cigarette smoke (CS) is a leading known cause of cancer and cardiovascular diseases worldwide. The mechanisms by which CS produces its damaging effects seem to be multifactorial. Among others, CS toxicity is due also to several compounds like $\alpha,\beta$-unsaturated aldehydes (acrolein, crotonaldehyde) and saturated aldehydes (acetaldehyde). Aldehydes could interact with thiol compounds of salivary proteins, leading to structural and functional alterations of these molecules. Prior *in vitro* studies have shown that there is a significant decrease in several known enzymatic activities following exposure to CS. Additionally, it was found that glutathione (GSH) has protective effect against the damaging role of CS to salivary enzymes, emphasizing the role of thiol groups in the mechanism of inactivation of these enzymes. In this study, salivary amylase activity showed a significant inhibition following exposure to CS, and to external addition of purified aldehydes known to be present in CS, due probably to the interaction between aldehydes and -SH groups of the enzyme. Our results indicate that although saturated aldehydes are the chief aldehydes present in CS, a significant decrease in amylase activity was due to unsaturated aldehydes, reacting, probably, through their double bond with the thiol group of proteins by the Michael addition reaction.

**Key word:** aldehydes, $\alpha$-amylase, cigarette smoke, glutathione, -SH groups.

**INTRODUCTION**

Cigarette smoke (CS) is presently known as the main cause of cancer, chronic bronchitis, emphysema, cardiovascular disease, and a leading cause of death worldwide (1). CS 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 (1-3).

CS contains over 4000 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, such as acrolein, crotonaldehyde (α,β-unsaturated aldehydes) and acetaldehyde (saturated aldehyde), all of which presumably are major causes of damage to various molecules exposed to CS (4).

The most abundant aldehyde in CS is acetaldehyde, and its concentration in CS is >20-80 times greater than that of other aldehydes present in gas phase cigarette smoke (1). Acetaldehyde is a known mutagen and carcinogen according to various in vitro cell culture studies and in vivo animal models. On the other hand, α,β-unsaturated aldehydes such as acrolein and crotonaldehyde are even more cytotoxic than are saturated aldehydes, such as acetaldehyde (5).

Saliva is an exceptional fluid, as it is the first biological fluid to encounter the inhaled CS gaseous and water soluble extracts. Previous studies have shown that aldehydes from CS are easily dissolved in the saliva during smoking (6). Hence, toxic aldehydes could mediate the carcinogenic effect of CS to oral cavity through saliva and from there, further on to upper respiratory and gastrointestinal tracts (1). Nagler et al. (4) have presented evidence that a portion of the damage to plasma proteins induced by CS was due to its aldehydes contained in it. In previous studies we have shown that exposure of saliva to plasma in vitro resulted in protein modifications, as measured by increased protein carbonyls (7). The source of the accumulation of protein carbonyls is attributed mainly to the volatile aldehydes present in CS (4). In addition, it has been shown that several salivary enzymes such as α-amylase, lactate dehydrogenase (LDH), and acid phosphatase are considerably affected by CS, while CS-based aldehydes, such as acrolein and crotonaldehyde, and oxygen free radicals were implicated as the causative agents affecting those enzymes (4, 7-9).

Saliva can be considered as one of the chief defense systems against aqueous soluble components of CS (11). As formerly reported, some components of the saliva could neutralize the toxic effect of aldehydes, oxidants, and carcinogens. Saliva contains nonenzymatic and enzymatic antioxidant systems, chiefly superoxide dismutase (SOD), glutathione peroxidase, uric acid, catalase, and glutathione (GSH) (10-13). According to recent studies (4), thiol compounds such as glutathione (GSH) and N-acetylcysteine can provide substantial protection from the CS associated loss of salivary amylase. GSH is possibly involved in the defense mechanism of saliva against noxious CS products, such as aldehydes which react with sulphydryl (-SH) groups. When protein -SH groups are involved, functional impairment of proteins and enzymes will occur. GSH could probably prevent this functional destruction as hypothesized in several in vitro studies, by making its -SH group available (10).

The purpose of the present study was twofold. Firstly, we sought to determine the interaction of toxic aldehydes with human saliva. Secondly, because thiols
have been proposed to have a protective effect against exposure to aldehydes, we examined the protective effects of GSH against aldehyde-induced saliva modifications, and we wished to elucidate the mechanisms whereby CS aldehydes might influence α-amylase activity.

MATERIAL AND METHODS

The study was approved by an institutional Ethics Committee and all volunteers gave their informed consent.

Collection of saliva

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 α-amylase activity studies were performed at the end of the CS and aldehydes exposure studies.

Cigarettes and aldehydes

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 was purchased from Sigma Chemicals Co., acetaldehyde from Fisher Scientific Co, and Crotonaldehyde from Aldrich Chemical Co.

Exposure of saliva to CS

An in vitro study was carried out using ‘Time’ cigarettes combined with a vacuum system, as described previously (4, 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 puffs. Samples for biochemical analysis were removed from the flasks at zero time and 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 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 (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.
α-amylase activity determination

The AMY assay method using Dimension® clinical chemistry system was employed in this project. The AMY method utilizes a chromogenic substrate, 2-chloro-4-nitrophenol linked with maltotriose. The direct reaction of α-amylase with the substrate results in the formation of 2-chloro-4-nitrophenol which is monitored spectrophotometrically. The AMY method responds to both pancreatic and salivary amylase isoenzymes. Enzyme activities were expressed as IU/L.

Thermochemiluminescence analysis (TCL)

Photon emission during heating was measured by TCL Photometer (manufactured by Lumitest, Israel) in the saliva, as previously described using a photomultiplier model R265P (Hamamatsu Photonics Co., Japan) with a spectral response range of 300-650 nm. The TCL device measures the level of photon emission from excited carbonyls in biological macromolecules, i.e., oxidative modifications of lipids and proteins. The fluid examined was distributed over the surface of an aluminum tray (a type of miniature Petri dish) inside the sample preparation block and was then vacuum dried. The dish is then mounted on a constant temperature heater kept at 80°C in the analysis block and the photon emission is measured. The TCL curve obtained is described mathematically by means of the two main parameters examined in the analysis (1) the amplitude of the kinetic curve of the photon emission and (2) its slopes. The TCL amplitudes both at 50 s (H1) and 280 s (H3) and the oxygenation potential and preincubation values were recorded, all representing the level of oxidative stress for which the saliva had been exposed to. H1 and H3 are base line and end point parameters of the TCL kinetic curve respectively that reflect electronically excited species formation due to heat induced oxidative modifications (14).

Addition of 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 Sigma Chemicals). In addition, in all experiments, a control without the compounds was run with no difference in the assay conditions.

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

Fig. 1 shows the loss of α-amylase activity after exposure of saliva of three volunteers to nine puffs of CS over a 3-h time period. It can be seen that α-amylase activity decreased over time by about 30% of the control level (P<0.001). The initial mean level from the three volunteers was 45x10³ ±8 IU/L. α-amylase activity did not change when saliva was incubated at 37°C under air. However, exposure of plasma to increasing numbers of puffs of gas phase CS produced a steady decrease in α-amylase activity. We received a similar pattern in response to CS, when α,β-unsaturated aldehydes, acrolein and crotonaldehyde,
were added. There was a reduction of 32% in comparison with the 4.7% reduction in the control (P<0.001). There was no statistical difference between CS and α,β-unsaturated aldehydes in reducing α-amylase activity. Acetaldehyde also reduced α-amylase activity in a significant manner in comparison with control (14% vs.

![Graph 1](image1)

**Fig. 1.** Effects of gas phase cigarette smoke (CS) on amylase activity in saliva. Salivary amylase activity was measured in the absence of CS or aldehydes - control (rhomb), in the presence of CS (square), crotonaldehyde & acrolein (AL; triangle), and acetaldehyde (circle). The results are expressed as means ±SD percentage of reduction in amylase activity during 3 h and are the means of eight separate experiments from 3 subjects. *P<0.001; see statistical explanation in the text.

![Graph 2](image2)

**Fig. 2.** Salivary TCL. Control on air (square), cigarette smoke (CS; rhomb), crotonaldehydes & acrolein (AL; circle) and to acetaldehyde (triangle). The results are expressed as means ±SD percentage of change in the TCL ratio during 3 h and are the means of eight separate experiments from 3 subjects. *P<0.001; see statistical explanation in the text.
5%; P<0.001), but in comparison with CS and α,β-unsaturated aldehydes it had significantly lower effect in decreasing α-amylase activity (14% vs. 33% and 32%; P<0.001 and P<0.02, respectively).

Fig. 2 shows changes in saliva TCL ratio. This ratio allows us to observe oxidative modification of proteins and lipids from time 0′ to 3 h. There was no change in TCL ratio when saliva was incubated at 37° C under air. Exposure of saliva to CS and α,β-unsaturated aldehydes produced a similar increase in TCL ratio (42% ±8 and 44% ±10, respectively). The increase was significantly different from the 2.0 ±1.5% control level (P<0.001). Acetaldehyde addition also produced a significant increase in TCL ratio which was significantly higher than the control (10.0 ±3.3% vs. 2.0 ±1.5%; P<0.001), but was lower than the effect of CS and α,β-unsaturated aldehydes (P<0.001). As mentioned, CS contains a number of constituents (e.g., oxidants, aldehydes) that can cause protein modifications and influence protein enzyme activity (15). To elucidate which constituents could cause loss of α-amylase activity, the saliva was exposed to CS and volatile aldehydes in the presence of 1 mM GSH.

Fig. 3 shows that the rate of loss of α-amylase activity was influenced by the presence GSH (33 ±11% for CS vs. 22 ±7% for CS+GSH; P<0.05) after 3 h. The addition of the major aldehydes present at concentrations contained in the CS of one cigarette influenced salivary α-amylase activity. Fig. 4 shows that in the setting of added α,β-unsaturated aldehydes, the rate of loss of α-amylase activity was also influenced by the presence of GSH (32 ±10% for α,β-unsaturated aldehydes vs. 14 ±3% for α,β-unsaturated aldehydes+GSH; P<0.001) after 3 h.
Fig. 5 shows that in the setting of added saturated acetaldehydes, the rate of loss of α-amylase activity was not significantly influenced by the presence of GSH (14 ±4% for acetaldehydes vs. 19 ±8% for +GSH; P<0.001) after 3 h.

Fig. 6 is a summary of all the experiments presented in this article. It shows the similar effects of CS and α,β-unsaturated aldehydes on amylase activity and...
that they both are influenced by the presence of GSH. It is worth noticing that the saturated aldehyde, acetaldehyde, had a much smaller effect on α-amylase activity in comparison with CS and α,β-unsaturated aldehydes (P<0.001; P<0.02) and that GSH had no influence on acetaldehyde-induced damage to α-amylase activity, as opposed to GSH profound protective effect against the toxicity induced by CS and α,β-unsaturated aldehydes.

DISCUSSION

Many noxious compounds in CS modify biological molecules by acting as oxidants, proinflammatory agents, or carcinogens (16-18). One type of such molecules are unsaturated and saturated aldehydes, present in CS (5, 13). All these toxic compounds first encounter oral cavity tissues, where saliva should act as a protector against the water soluble fraction of CS (10). In the present study, noxious effects of CS aldehydes have been evaluated in vitro directly in fresh human saliva samples. The major aim of the study was to try to understand the underlying causes behind the phenomenon that gas-phase CS can rapidly inactivate a large percentage of α-amylase activity in vitro. Firstly, we examined the degree to which volatile aldehydes present in CS could account for the effects of CS on saliva through α-amylase activity and carbonyl formation. Secondly, we examined the ability of GSH to amend the effects of these aldehydes in fresh human saliva.

Previous studies have shown that the aldehydes of CS gas phase could explain partially some of the protein modification caused by CS. Protein modification was examined through carbonyl formation and enzymatic activity. Reznick et al (7) and
O’Neill et al (5) have shown that exposure of plasma proteins to CS or directly to aldehydes induced their modification as evidenced by the enhancement of plasma carbonyl groups and a parallel decrease in -SH groups. In the present saliva study, the effects of CS and volatile aldehydes on protein carbonyls resemble, to a great extent, those found in plasma studies (7). Exposure of saliva to a mixture of purified unsaturated aldehydes (crotonaldehyde and acrolein), at concentrations known to be present in CS, resulted in a similar increase of protein carbonyls to CS, measured by TCL technology, which measures excited carbonyls (5). On the other hand, the saturated aldehyde, acetaldehyde, which is a major volatile constituent of the CS and present in CS in relatively high concentrations, had a less significant effect on carbonyl formation, as opposed to CS and unsaturated aldehydes (Fig. 2).

A more extensive study was performed in order to understand the factors involved in α-amylase inactivation due to CS exposure. Exposure of saliva to CS significantly accelerated α-amylase loss of activity. A similar pattern was shown by the addition of unsaturated aldehydes, and, to a much less extent, by acetaldehyde (Fig. 1).

GSH

In previous studies, functional modifications induced by CS were partially prevented by adding to the incubation system sulphydryl compounds, like GSH, whose role could be to ‘offer’ –SH groups to aldehydes instead of proteins (10). In other studies, where purified aldehydes were shown to cause accumulation of protein carbonyls in plasma (10), GSH was capable of ameliorating this accumulation in both plasma and salivary proteins (4, 10). In the present study, GSH slowed down the rate of CS-associated loss of α-amylase activity (Fig. 3). A comparable influence was found when saliva was incubated with GSH before the addition of unsaturated aldehydes (Fig. 4). There was no significant influence in the case of acetaldehyde (Fig. 5). GSH did not completely inhibit the CS and unsaturated aldehydes decrease of α-amylase activity. However, GSH slowed this loss considerably. This may suggest that GSH provides protection against α-amylase oxidation, while protecting some protein -SH groups, probably at the active site of the enzyme. Indeed, the role of -SH groups in preserving α-amylase activity has already been shown (4).

The present study indicates that aldehydes present in CS, especially unsaturated aldehydes, contribute significantly to protein modification caused by CS and that thiols appear to be protective against aldehyde-induced protein modification as measured by the end points of excited carbonyl formation (TCL) and α-amylase activity. It is likely that thiols such as GSH are potentially beneficial in protecting proteins against the toxic effects of unsaturated aldehydes.

Proposed mechanism

The results of the present study suggest that the unsaturated aldehydes in CS are the major source of reactive double bonds reacting with -SH groups of proteins
in the Michael addition reaction, attaching the aldehydic carbonyls to the proteins (4, 5). Indeed, in previous studies in plasma, it was shown that a 1:1 stoichiometry, on a molar-to-molar basis, exists between the appearance of protein carbonyls and the disappearance of protein thiol groups (5). Thus, it is quite reasonable to assume that also in the case of saliva, the increase of protein carbonyls, due to exposure to CS, is mainly due to reaction of unsaturated aldehydes with protein -SH groups, adding their carbonyls attached to proteins (4).

The mechanism of protein damage by saturated aldehydes is more bewildering. It is possible that these aldehydes react with proteins, forming adducts at both -SH and -NH₂ groups, but it is unlikely that these adducts will contain carbonyl groups. Therefore, the increase in carbonyl formation after the addition of acetaldehyde was significantly lower in comparison with CS and unsaturated aldehydes.

The reduction of aldehyde-induced protein modification by GSH may stem from the added -SH moieties that are available to react with aldehydes. Indeed, the addition of GSH to saliva, may reduce the concentration of mixed disulfides, thus increasing the amount of protein-SH moieties, suggesting that unsaturated aldehyde-induced protein damage could be ameliorated by augmented GSH (5).

The results of our study clarify the underlying mechanisms of the immediate and overwhelming induced inactivation of α-amylase by CS and aldehydes, and of partial protection rendered by GSH against it. Our data show that the addition to saliva of a mixture of aldehydes found in CS leads to the formation of products that are detectable in the carbonyl assay and cause some inactivation of α-amylase. Therefore, we conclude that at least some of the carbonyl formation and α-amylase inactivation caused by CS is due to aldehydes, chiefly unsaturated aldehydes.

The loss of α-amylase activity and carbonyl formation reported in the present study, along with other studies due to CS and aldehydes, could play an important role in the initiation and progression of oral inflammatory diseases, promote premalignant and malignant lesions of the oral mucosa, and destroy normal homeostasis of the oral cavity. We found that treating the whole saliva with antioxidant thiols, such as glutathione, shows that GSH might be a significant protector against CS and/or unsaturated aldehyde-induced damage. Further studies are needed to elucidate the role of CS-related inhibition of saliva α-amylase activities in oral pathobiology. If corroborated by further studies, these findings may help develop an effective means of protecting against CS-associated damage to salivary enzymes and proteins.

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