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Lethal synergistic effect of cigarette smoke and saliva in an *in vitro* model: does saliva have a role in the development of oral cancer?

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Abstract

Exposure of oral mucosal cells to cigarette smoke induces oral cancer, presumably via the injurious effect of free radicals. To explore the effects of cigarette smoke on cells in the presence of saliva, we used peripheral blood lymphocytes (PBL) and exposed them to cigarette smoke, alone or in the presence of saliva. After 80 min exposure to cigarette smoke alone, a time-dependent cellular loss and survival rate of 52% was observed. By contrast, following the exposure of the lymphocytes to cigarette smoke in the presence of saliva, less than 20% of the cells survived. Saliva secreted from the submandibular/sublingual (Sm/Sl) glands was highly cytotoxic, while saliva secreted from the parotid glands was only moderately cytotoxic. Redox active iron ions in saliva and aldehydes in cigarette smoke were shown to play the major injurious roles in this synergistic phenomenon. The salivary-borne redox active iron ions participate in Fenton and Haber–Weiss reactions to transform low-reactive free radicals, which originate from cigarette smoke into highly-reactive $^{\cdot}OH^{-}$-free radicals. In light of these results, a comprehensive mechanism for the induction of oral cancer by cigarette smoke is suggested where saliva may be a pivotal player.

Keywords: Oral cancer; Aldehydes; Oxidative stress; Free radicals; Saliva; Cigarette smoke; *in vitro*

1. 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 characterised by a high rate of patient morbidity and mortality (over 50%), similar to the rates observed for malignant melanoma [1–4]. The main inducing agent of oral SCC is tobacco, which is considered to be responsible for up to 90% of the cases worldwide and may explain why this cancer is more prevalent in men [5–7]. Further, cigarette smoking has been identified as the most avoidable causative agent with regard to mortality rates in the United States [8]. The prevalence of oral SCC in cigarette smokers is 4–7 times higher than in non-smokers, and when alcohol or chewing tobacco habits are also present, the disease prevalence increases by 19- and 123-fold, respectively [6]. The cigarette smoking-related higher risk for oral SCC is also emphasised by a reduction in the mean age of disease presentation of 15 years [9,10]. Currently, the ''field cancerisation'' concept is widely accepted as an explanation for the carcinogenic effect of cigarette smoke on the oral mucosa. Various cigarette smoke-borne agents constantly and directly attack the oral epithelial cells, resulting in gradual accumulation of mutations and step-wise malignant transformation. These agents primarily include free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). The transformation process gradually progresses from dysplastic lesions of the mucosa to lesions of carcinoma *in situ* and eventually result in a full-blown infiltrating and metastasising oral SCC [11]. Similarly, premalignant oral epithelial lesions, such as lichen planus or leucoplakia, may progress to oral cancer following

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insult by free radicals resulting in the induction of cellular and molecular alterations [12]. Indeed patients with oral lichen planus have a lower salivary antioxidant capability making them more susceptible to such insults [13]. The possible role of saliva as an oral anti-carcinogenic was demonstrated recently in oral SCC induced by a local carcinogen (4NQO) [14]. In this animal study, Dayan *et al*. showed saliva significantly inhibited the initiation and progression of oral SCC. Further, Nishioka *et al*. [15], using the Ames test, found that saliva inhibited the mutagenicity of another well-known local oral cancer inducer (benzopyrene). Such anti-carcinogenic features may be attributed to antioxidant systems in saliva which have recently been elucidated [16]. However, in contrast to these reports, we recently found that the interaction between cigarette smoke and saliva may be deleterious (rather than affording protection) resulting in the rapid destruction of biological macromolecules, such as enzymes and proteins [17,18]. In these studies, we also demonstrated that aldehydes and redox active metal ions mediate this destructive consequence of the interaction between cigarette smoke and saliva. Given mutagenic alterations of the oral mucosal cells induced by cigarette smoke must occur in the presence of saliva and lymphocytes have previously been shown to be very sensitive to free radical-mediated injuries, such as exposure to ionising irradiation or hydrogen peroxide [19,20]. To clarify further these seemingly contradictory results, we exposed peripheral lymphocytes to cigarette smoke, alone or in the presence of saliva.

2. Materials and methods

2.1. Collection of saliva

Whole saliva, parotid and Sm/Sl saliva were collected from six healthy volunteers (three males and three females, age range 21–47 years) under non-stimulatory conditions in a quiet room between 8 a.m. and noon. Collection was performed at least 1 h after eating. For whole saliva collection, patients were asked to generate saliva in their mouths and to spit into a wide test-tube for 10 min, while parotid and Sm/Sl saliva were collected as previously described in [21]. Following collection, the saliva was immediately centrifuged at 800*g* at 4 °C for 10 min to remove squamous cells and cell debris. The resulting supernatant was used for the biochemical analysis.

2.2. Lymphocyte isolation

Blood from 10 consenting, healthy, non-smoking volunteers (five males and five females, age range 18–55 years) was drawn into ethylene diamine tetra-acetic acid (EDTA) containing vacutainers. Human peripheral blood lymphocytes (PBL) were prepared using a Ficoll-Hypaque (Sigma) gradient centrifugation according to the manufacturer's instructions. Lymphocytes were suspended in Phosphate-Buffered Saline (PBS, Beit-Ha'emek Industries, Israel) to a density of 10×10^6, and used immediately.

2.3. Exposure of lymphocytes to cigarette smoke in medium (PBS) with/without saliva

The cigarettes used in this study were commercial Time cigarettes containing 14 mg of tar and 0.9 mg of nicotine (Time Cigarettes, Dubek Ltd., Tel Aviv, Israel). A time cigarette (capable of removing particles), in which the filter tip was removed, was attached to a Cambridge filter and this was combined with a vacuum system to draw gas-phase cigarette smoke inside sealed 250 ml flasks that contained lymphocytes in 12–15 ml PBS, as previously described in [17,18]. A reproducible vacuum was created in the flask. Upon opening the vacuum to the lighted cigarette for 5 s, 80–100 ml of cigarette smoke "puffs" were drawn into the flask. Whenever mentioned, thiols glutathione (GSH) or *N*-acetyl-L-cysteine (NAC), desferal (DES), ascorbate (Asc) or $FeCl_3$ (Sigma) were added at the concentrations mentioned. Where needed, the lymphocyte-containing PBS was supplemented with 30% (v/v) saliva. After half the cigarette was used, the flasks were incubated for 20 min at 37 °C in a metabolic shaker, and then the procedure was repeated four times.

2.4. Survival of lymphocytes

The viability of the lymphocytes was measured at various time points using a Trypan Blue exclusion test, both in the exposed and control cells.

2.5. Statistical evaluation

Means, standard deviations and standard errors were computed. Results between the subgroups were analysed with a "One-way analysis-of-variance" [23] using the Bonferroni multiple-comparison test model [24]. The Bonferroni model tests the hypothesis that the means from all subgroups are equal or significantly different. The means between two subgroups of patients were analysed using the two sample *t*-test for differences in means [25].

3. Results

3.1. Exposure of lymphocytes to cigarette smoke and/or saliva

During an 80-min incubation period of lymphocytes in PBS alone or in PBS supplemented with 30% (v/v) saliva, no loss in survival occurred (Fig. 1). However, 80 min exposure of the lymphocytes (in PBS) to cigarette

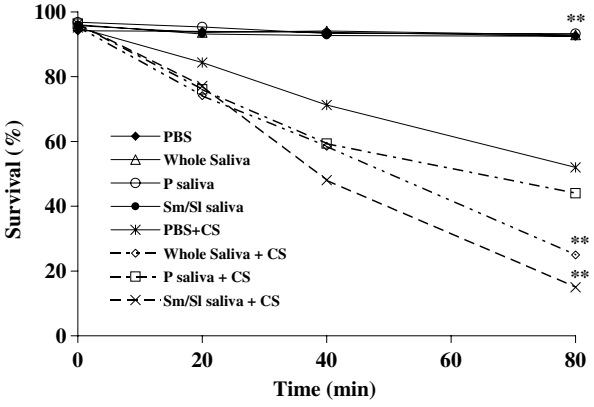

Fig. 1. Survival rate of peripheral blood lymphocytes (PBL) cells exposed to cigarette smoke (CS) and differentiated saliva, $P < 0.01(**)$. PBL incubated at 37 °C in PBS alone. PBL incubated at 37 °C in the presence of whole saliva. PBL incubated at 37 °C in the presence of parotid (P) saliva. PBL incubated at 37 °C in the presence of sub mandibular/sublingual (Sm/Sl) saliva. PBL incubated at 37 °C in PBS alone and exposed to cigarette smoke. PBL incubated at 37 °C in the presence of whole saliva and exposed to cigarette smoke. PBL incubated at 37 °C in the presence of parotid (P) saliva and exposed to cigarette smoke. PBL incubated at 37 °C in the presence of Sm/Sl saliva and exposed to cigarette smoke. Please note that all groups are composed with PBS and CS group out at 80 min only.

smoke resulted in a time-dependent reduced survival of the cells. This was most significant after 80 min (survival loss of 48%, $P < 0.01$). The addition of whole saliva to the lymphocytes, while exposing them to cigarette smoke resulted in a lethal synergistic effect, as demonstrated by a 82% ($P < 0.01$) loss in survival. Fig. 1 also shows the survival results obtained for lymphocytes exposed to cigarette smoke with/without two other types of saliva – parotid and Sm/Sl saliva, collected under resting conditions. As is clearly noted, both parotid and Sm/Sl saliva also induced this lethal synergistic effect. Parotid saliva significantly enhanced the lethal effect of cigarette smoke when added to the lymphocytes for 40 min by 56% ($P < 0.05$), but not when added for 80 min. The Sm/Sl was found to be the most cytotoxic saliva examined and its synergistic effect was significantly more lethal both at 40 and 80 min exposure. At 40 min exposure, the Sm/Sl saliva enhanced the lethal effect of cigarette smoke by 98% ($P < 0.01$). At 80 min, while the survival rate of the cells following exposure to cigarette smoke in the presence of lymphocytes in PBS alone was reduced by 48%, in the presence of medium supplemented with whole saliva and medium supplemented with Sm/Sl saliva, it decreased by 82% and 89%, respectively, ($P < 0.05$).

3.2. Modification of cigarette smoke and/or salivary effects by antioxidants

The addition of 1 mM ascorbate or 1 mM NAC did not alter the loss of cell survival following their exposure to cigarette smoke and saliva. In contrast, addition of 1 mM GSH partially prevented this lymphocyte death. Thus, at 40 and 80 min following exposure to cigarette smoke and saliva, the survival rates dropped to 55% and 14%, respectively, while in the presence of GSH, these survival rates were 91% ($P = 0.0001$) and 23% ($P = 0.0037$), respectively (Fig. 2). The demonstrated protective effect by GSH led us to hypothese that cell death was mediated by the action of aldehydes originating from cigarette smoke. In contrast to GSH, addition of 1 mM DES did not protect the lymphocytes at 40 min. However, at 60 min exposure, DES partially protected the cells (not statistically significant) while at 80 min DES significantly protected the cells (survival rate 22% ($P = 0.005$) Fig. 2), similar to the protection afforded by 80 min incubation with GSH.

3.3. Role of transition metal ions

The fact that DES had a similar protective effect to GSH (although only at later time-points), points to a possible role for redox active iron ions. To test this hypothesis, we employed a commonly-used assay to examine whether metal ions in a biological milieu are indeed redox-active. This assay is based on the established *pro-oxidant activity* of ascorbate in the presence of redox-active ions. We also examined the possible role of iron ions using DES, a very potent iron chelator. We found that the addition of ascorbate to saliva-containing medium (without exposing it to cigarette smoke) resulted in a reduction in the lymphocyte survival rate to 75% ($P < 0.01$) (Table 1). The modulatory role of iron was further demonstrated by the direct addition of ascorbate to iron ($FeCl_3$) (in a PBS medium which did

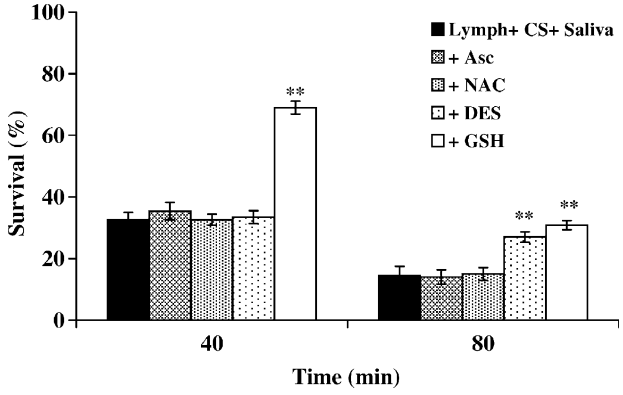

Fig. 2. The effects of several antioxidants on the survival of human PBL exposed to cigarette smoke and saliva, $P < 0.01(**)$. Lymph, lymphocytes. PBL incubated at 37 °C in the presence of cigarette smoke and saliva. PBL incubated at 37 °C in the presence of cigarette smoke, saliva and 1 mM ascorbate (Asc). PBL incubated at 37 °C in the presence of cigarette smoke, saliva and 1 mM *N*-acetyl-L-cystein (NAC). PBL incubated at 37 °C in the presence of cigarette smoke, saliva and 1 mM desferal (DES). PBL incubated at 37 °C in the presence of cigarette smoke, saliva and 1 mM glutathione (GSH).

Table 1
The survival of human PBL exposed to saliva, several antioxidants and redox-active iron at 80 min (without CS)

		Obs.	Mean (%)	St.Err
1	Lymph + PBS	3	96	4
2	+Saliva	3	96	4
3	+Asc 1mM	3	96	4
4	+DES 1mM	3	95	3
5	$+Fe^{3+}$ 90 μM	3	91	5
6	+Saliva + Asc	3	75	5
7	+Saliva + Asc + DES	3	93	4
8	$+Fe^{3+}$ + Asc	3	72	4
9	$+Fe^{3+}$ + Asc + DES	3	94	5

CS, cigarette smoke; Obs, observations; St.Err, standard error; PBS, phosphate-buffered saline; DES, desferal; PBL, peripheral blood lymphocytes; Asc, ascorbate; Lymph, lymphocytes.

not contain saliva). This addition also resulted in reduction in the lymphocyte survival rate to 72% (Table 1), which was totally prevented when DES was added to the system. Interestingly, neither ascorbate nor DES had any modulatory effect on the lethal effect that cigarette smoke had on cells in the *absence of saliva* (58% survival rate) (Table 2). This proved that the redox active iron ions did not originate from cigarette smoke, but rather from saliva.

3.4. Protection by GSH and DES against the effects of cigarette smoke and saliva on lymphocytes

Having identified two major underlying mechanisms involved in the cell death in our system, i.e., the action of aldehydes and highly reactive free radicals mediated by redox-active iron, we initiated a study to try to protect the lymphocytes against both injurious mechanisms simultaneously. We added GSH to the lymphocytes exposed to cigarette smoke and saliva alone (1 mM) or concomitantly with DES, in either low (1 mM) or high (5 mM) concentrations. Survival rates were examined at 20 and 80 min (Fig. 3). We found the addition of 1 mM GSH and 5 mM DES to the saliva-containing medium prior to exposure to cigarette smoke afforded excellent protection against the lethal effects of cigarette

Table 2
The survival of human PBL exposed to CS, ascorbate and DES at 80 min (without saliva)

		Obs.	Mean (%)	St.Err
1	Lymph	3	96	6
2	+CS	3	58	3
3	+Asc 1 mM	3	96	6
4	+DES 1 mM	3	95	5
5	+CS + Asc	3	66	4
6	+CS + Asc + DES	3	60	5

CS, cigarette smoke; DES, desferal; Asc, ascorbate; Lymph, lymphocytes.

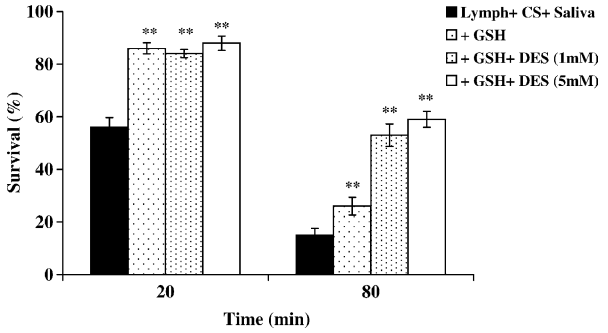

Fig. 3. Mean survival rate ± standard error of the mean (SEM) of lymphocytes exposed to cigarette smoke in the presence of saliva at 20 min and at 80 min. These survival rates were compared with those obtained when either 1 mM GSH alone or in combination with 1 or 5 mM DES were added to the medium prior to exposure to the cigarette smoke, $P < 0.01(**)$.

smoke. While survival rates of the control cultures of were 56% and 15%, at 20 and 80 min, respectively, these rates became significantly higher ($P = 0.0001$) in cultures incubated with the protective agents; 88% and 59%, for GSH (1 mm) and DES (5 mm), respectively.

4. Discussion

The most surprising result in our study was the rapid, synergistic and lethal effect that exposure to cigarette smoke in the presence of saliva had on peripheral lymphocytes. This in spite of the natural salivary antioxidant capacity (when not in the presence of cigarette smoke) in which uric acid is the key molecule, while peroxidase is the major antioxidant enzyme [16,26,27].

Saliva secreted by the Sm/Sl glands was found to be highly cytotoxic compared with saliva secreted by the parotid gland. One explanation may be that most of the balancing antioxidants in saliva, such as peroxidase, superoxide dismutase and uric acid, are secreted by the parotid and not the Sm/Sl glands, as was recently demonstrated in [16]. Accordingly, the injurious agents which produce the lethal oxidative stress, can not be neutralised in Sm/Sl saliva and are, consequently, very harmful. Worringly, Sm/Sl saliva is by far the most important maintenance saliva and is secreted in large volumes at all times. By contrast, parotid saliva is secreted mainly following stimulation, such as during ingestion. Accordingly, the oral mucosa is bathed by Sm/Sl saliva most of the time and thus damage to the cells may occur whenever a cigarette is smoked.

We have recently demonstrated in humans that Sm/Sl saliva contains much higher concentrations of iron than parotid saliva, especially when secreted under resting conditions. This could help to explain some of its higher cytotoxicity compared with parotid saliva.

The exact mechanism responsible for the saliva and cigarette smoke induced lymphocyte death has yet to be

elucidated. However, modifications of proteins, rendered by free radicals inducing carbonylation [28,29] may be one mechanism that leads to the destruction of protein structures in the cellular membrane. In any case, no lymphocyte loss in the presence of saliva only (without cigarette smoke), was observed.This is not surprising as saliva is considered a harmless medium that is 'armed' with various protective capabilities, from different enzymatic, immunological and antioxidant defense systems [30,31]. The importance of saliva's antioxidant capacity was clearly demonstrated by Nair *et al*. [32] who reported that saliva inhibited the production of radical oxygen species, the superoxide free radical ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) from betel quid tobacco, the most potent inducer of oral cancer. This may be attributed to the antioxidant components of saliva in which uric acid, a very potent antioxidant, is a key player [33,34]. However, in the presence of cigarette smoke, saliva had the opposite effect in our study and doubled the cigarette smoke-induced lethal effect. Within 80 min, over 80% of the lymphocytes were lost. The synergistic effect of cigarette smoke and saliva could be important in understanding the well-described devastating effects that cigarette smoke has in the oral cavity, where both cancer and inflammatory diseases such as periodontitis are induced.

It could be argued that oral epithelial cells would be a better model to use rather than lymphocytes. However, these cells are technically much more difficult to use and are not as sensitive to oxidative stress. Thus, as we were evaluating the principle of an increase in the oxidative stress level of the cell the lymphocyte model seemed the most appropriate to use. Lymphocyte sensitivity results from their very low transport activity for L-cystine resulting in a low baseline supply of GSH precursors [35–37]. As a consequence, lymphocytes are highly susceptible to changes in the intracellular redox state. Cytoplasmic GSH levels are largely determined by the availability of its precursor L-cysteine, and the most important source of L-cysteine in plasma is L-cystine, the disulfide-linked oxidised dimer [35–37]. The protective capacity of GSH is probably mediated via inhibition of nuclear factor κB (NFκB), which is a transcription factor activated by the excessive cellular production of reactive free radicals.

Traditionally, the effects of cigarette smoke are thought to be mediated by a direct attack of ROS and RNS on the surrounding biological macromolecules and cells. To further explore the synergistic relationships between cigarette smoke, and saliva we employed two lines of research:

(A) We added various antioxidants to the system in an attempt to reduce the induced cell damage and thus detect any mediatory roles for the various oxidants;
(B) We added various oxidants to the lymphocytes without either cigarette smoke or saliva in an attempt to mimic the induced damage and again to reveal any mediatory roles for the oxidants.

Both these lines of research revealed two major mechanisms acting in concert leading concomitantly to the cell damage observed. One mechanism involves cigarette smoke-borne aldehydes directly attacking cells and is unrelated to the salivary effects of the cells. The second mechanism involves an attack by aggressive ROS, whose production is linked to active iron ions in saliva. The cell damage mediated by aldehydes, accords well with report by Reznick *et al*. [22,38,39] in the early 1990's where severe aldehydes-mediated damage to plasma proteins was observed. Further credence for this role played by aldehydes is provided by the fact that the most important biological thiol, GSH, partially prevented lymphocyte-death at all time points examined. NAC, another thiol and a non-toxic GSH-prodrug, did not protect the lymphocytes and this may be explained by the fact that it is readily taken up by the cells, while GSH is not. Thus, GSH is left outside the cell and can neutralise the aldehydes from the cigarette smoke [35–37]. We proposed a synergistic mechanism based on three observations:

(A) The addition of DES partially prevented the cell damage, although only at later time points. This may suggests that there is some kind of "cross-talk" between the two protective pathways. The aldehyde-mediated attack may pave the way for a later attack inflicted by the salivary iron-related ROS. While DES protection was not significant at the earlier time points, at 80 min its protective capacity was as efficient as that of GSH.
(B) The damage was partially mimicked by replacing cigarette smoke with ascorbate and adding it to the saliva.
(C) The damage was also partially mimicked to a similar level, by adding iron and ascorbate to the lymphocytes, in the absence of either saliva or cigarette smoke.

All these findings point to a role for redox-active iron which originates in saliva, as it is known that redox-active iron in the presence of ascorbate participates in Fenton and Haber–Weiss reactions to transform low-reactive free radicals into highly-reactive $\cdot OH^-$ free radicals. The preventive role of DES added to the saliva prior to its exposure to cigarette smoke supports this conclusion, as DES is considered a very potent iron chelator. Further credence for the suggested mechanism is found in the fact that neither ascorbate nor DES altered the lymphocyte loss rate rendered by cigarette smoke alone, which may be explained by the assumption that redox-active iron does not originate from cigarette smoke, but only from saliva. The demonstration of the existence of redox active metals, iron and copper, in parotid saliva and their deleterious role in the pathogenesis of ionising irradiation-induced damage to this

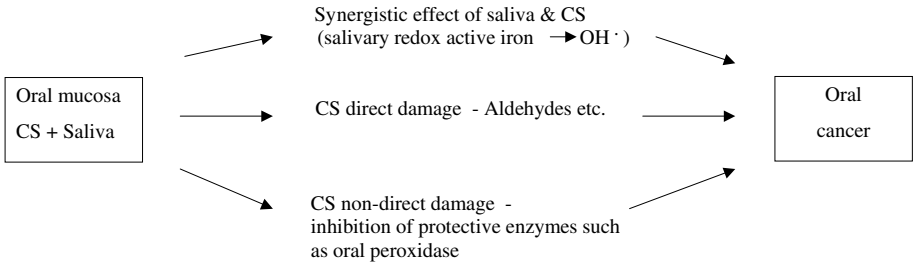

Fig. 4.

gland, as demonstrated by Nagler *et al.* [19,20] in the late-1990s provides further support for our hypotheses.

Cigarette smoke-induced injury to lymphocytes has been previously shown by others, who demonstrated by cellular anergy, various losses in cell function, genomic deletions and other DNA injuries such as a decreased number of micronuclei or DNA adduct formation [40–44]. In this respect, the study published by Yang *et al.* [45] is especially important. They also found that exposure of human lymphocytes to water-soluble compounds, from cigarette smoke, resulted in DNA damage, as revealed by a COMET assay. The rapidly induced DNA damage was attributed to directly acting compounds from cigarette smoke. Interestingly, it was also enhanced by ascorbate, probably by redox cycling of low-reactive free radicals present in cigarette smoke. Furthermore, Yang and colleagues also reported that, acrolein (a highly prevalent aldehyde in cigarette smoke) induced DNA damage. Moreover, the suggested dual mechanism is further supported by the fact that both metal-related ROS and aldehydes have previously been shown to alter protein structure and function [28,29,39]. However, we believe that the most significant proof for the suggested dual mechanisms of lymphocyte death was shown by our ability to substantially prevent cellular death. This was achieved by the concomitant addition of high concentrations of both GSH (1 mM) and DES (5 mM) to the saliva prior to exposing the lymphocytes to cigarette smoke.

Our results may contribute substantially to our understanding of the process leading to oral cancer. Up to 90% of oral cancers are related to tobacco products; cigarette smoke in the Western world and betel quid in South East Asia [1–4,7]. As most cases occur in South East Asia [46], two studies reporting [32,47] that while saliva inhibited the production of free radicals from betel quid, the *in vitro* addition of active metal ions iron and copper enhanced the production of free radicals from the betel nut are important. We also found that saliva in the presence of cigarette smoke, dramatically enhanced the level of free radical production and, consequently, the lymphocyte killing rate. We concluded that the death rate of over 80% resulted from both the severe, lethal nature of the aldehydes and the metal-related ROS attack, and from the extreme sensitivity of the lymphocytes to free radical attack. However, oral epithelial cells are not as vulnerable to attack by free radicals, and the effect on these cells is not expected to be as severe and may be sublethal only, leading to DNA damage, mutagenic transformation and the induction of cancer. In this respect, the afore mentioned reports of DNA damage induced by cigarette smoke on lymphocytes may be very significant [40,42,45], as well as the recently reported impairment of neutrophil respiratory burst activation by aldehyde-induced thiol modification [48]. A schematic for of the suggested mechanism of the process leading to oral cancer is depicted in Fig. 4. It is based on both cigarette smoke-induced salivary-related and -unrelated injurious factors, which act, either directly or indirectly to cause mutagenic aberrations. These mechanisms are also supported by many studies demonstrating that the most widely used chemopreventive agents against oral pre-cancer (in humans) and oral cancer (in both in *in vitro* and *in vivo* models) are GSH and antioxidants (vitamin 5 A, E, β-carotene) and free radical scavengers [2,49–54]. Moreover, in contrast to other types of cancer in which ascorbate (vitamin C) acts as a protector, it was demonstrated that vitamin C in oral cancer enhanced the development of carcinomas in an *in vivo* hamster buccal pouch experimental model, while it protected oral cancer cells in an *in vitro* model, where there was *no saliva* and, consequently, *no active metal ions* [55,56].

In summary, a comprehensive mechanism for the induction of oral cancer by cigarette smoke is suggested by our results whereby saliva loses its antioxidant capacity and becomes a potent pro-oxidant milieu in the presence of cigarette smoke (Fig. 4). Our data suggests saliva may be a pivotal player in the process leading to oral cancer.

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