Journal section: Oral Medicine and Pathology
Publication Types: Research

Synergistic effects of cigarette smoke and saliva

Yalda Nozad-Mojaver [1], Maysam Mirzaee [2], Abdullah Jafarzadeh [3]

[1] Assistant professor, Department of Oral Medicine, Rafsanjan University of Medical Sciences, Dental school, Rafsanjan, Iran
[2] D.D.S. Dentist
[3] Associate Professor of Immunology, Department of Immunology, Medical School, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

Correspondence:
No # 8, Fourth alley, Kadge sq,
Saadat-Abad, Tehran, IRAN,
mojaver@farabi.tums.ac.ir

Received: 31/07/2008
Accepted: 15/02/2009

Nozad-Mojaver Y, Mirzaee M, Jafarzadeh A. Synergistic effects of cigarette smoke and saliva. Med Oral Patol Oral Cir Bucal. 2009 May 1;14 (5):E217-21.
http://www.medicinaoral.com/medoralfree01/v14i5/medoralv14i5p217.pdf

Article Number: 5123658847 http://www.medicinaoral.com/
© Medicina Oral S. L. C.I.F. B 96689336 - pISSN 1698-4447 - eISSN: 1698-6946
eMail: medicina@medicinaoral.com
Indexed in:
-SCI EXPANDED
-JOURNAL CITATION REPORTS
-Index Medicus / MEDLINE / PubMed
-EMBASE, Excerpta Medica
-SCOPUS
-Indice Médico Español

Abstract
Objectives: The aim of this study was to evaluate the cytotoxic effects of Cigarette Smoke on the human peripheral blood lymphocytes in the presence of stimulated or non-stimulated saliva in an in vitro model.
Methods and Materials: Ten healthy volunteers in the age range of 21 to 29 were selected and samples of peripheral blood lymphocytes and saliva (whole and stimulated saliva) collected. Peripheral blood lymphocytes suspensions (PBS) were taken and exposed to 6 different media. Samples were collected from all media at both 20 and 80 minute time points. The survival rates of PBL were then determined at both 20 and 80 minute time points.
Results: The cell survival rates following exposure to cigarette smoke (CS) in the presence of PBS supplemented with whole or stimulated saliva were significantly lower at 80 minutes when compared with the 20 minute rates (P<0.05).
Conclusion: Tobacco effects were significantly increased in the presence of saliva especially stimulated saliva.

Keywords: *Cigarette smoke, whole saliva, stimulated saliva, in vitro*

Introduction
Saliva is a most valuable oral fluid and yet it is often taken for granted. It is critical to the preservation and maintenance of oral health, and yet it receives little attention until quantity or quality is diminished.
Nowadays, one-third of adults (1.3 billion people) are known to be smokers. Over 4000 bioactive chemical compounds have been isolated from cigarette smoke, of which more than 300 carcinogens have been identified in smoke or in its water-soluble components that will leach into saliva (1). 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 (2). 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 (2). However, there is never a direct contact between cigarette smoke (CS) and the oral mucosa. Saliva, bathing the mucosa from the oral cavity to the larynx, always intervenes, and CS must first interact with saliva before it reaches the mucosa. More-

over, Bloching et al. found increased genotoxic activity in the saliva of smokers with a highly significant additional increase of genotoxicity measured in smoking and drinking individuals (3).
The possible role of saliva as an oral anti-carcinogenic agent was demonstrated recently in oral SCC induced by a local carcinogen by Dayan et al. (4). They showed saliva significantly inhibited the initiation and progression of oral SCC. Further, Nishioka et al. (5) 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 (6). Indeed patients with oral lichen planus have a lower salivary antioxidant capability making them more susceptible to such insults (7). However, in contrast to these observations, it has recently reported 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 (8). The oral mucosa is bathed by saliva and thus damage to the cells may occur whenever a cigarette is smoked. In fact, that mutagenic alteration of the oral mucosal cells induced by CS must occur in the presence of saliva. In this study, peripheral lymphocytes were exposed to cigarette smoke, alone or in the presence of stimulated or whole saliva, since lymphocytes have previously been shown to be so sensitive to free radical-mediated injuries, such as exposure to irradiation or hydrogen peroxide (9).
To evaluate the synergic effects of cigarette smoke and saliva, we exposed peripheral lymphocytes to cigarette smoke, alone and in the presence of whole and stimulated saliva.

Material and Methods

2.1. Collection of whole saliva (un-stimulated saliva)

An interventional prospective study on a number of healthy volunteers who had signed an informed consent over the period from September to November 2006. Whole saliva, were collected from ten healthy dental students (five males and five females, age range 21–29 years) under non-stimulatory conditions in a quiet room between 10 to 11 am . All participants refrained from eating and drinking for a minimum of 1 hour before saliva collection. During this period, participants were seated in a relax position and after a few minutes of relaxation, they were trained to avoid swallowing saliva and asked to lean forward and spit all the saliva they produced for 5 ml into a graduated test tube, through a glass funnel.

2.2. Collection of stimulated saliva

15 minutes after collecting the whole saliva, 0.2 ml of sour lemon juice was dropped onto the posterior surface of the tongue. Participants were asked to generate saliva in their mouths and to spit saliva into a graduated test tube until 5 ml again.
Following collection, the whole and stimulated saliva was immediately centrifuged at 800g at 4 °C for 10 min to remove squamous cells and cell debris. The resulting supernatant was used for the biochemical analysis

2.3. Lymphocyte isolation

Blood from 10 consenting, healthy, non-smoking volunteers (five males and five females, age range 21-29 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 1×106 cells/ml PBS in wells of micro plate and used immediately.

2.4. Lymphocytes exposure

After separation of PBL, lymphocyte suspensions (PBS) were produced (1×10^6 cell/ml in PBS). For better evaluation, six media were prepared from PBS and cigarette smoke as follows:

1. Lymphocyte suspension alone (control group 1)
2. Lymphocyte suspension + whole saliva (Control group 2)
3. Lymphocyte suspension + stimulated saliva
4. Lymphocyte suspension + cigarette smoke
5. Lymphocyte suspension + cigarette smoke + whole saliva
6. Lymphocyte suspension + cigarette smoke + stimulated saliva

The cigarettes used in this study were commercial Winston cigarettes containing 14 mg of tar and 1 mg of nicotine (Winston Red Cigarettes, R.J. Reynolds Tobacco Company, USA).Cigarettes, in which the filter tip was removed, was attached to a Cambridge filter and this was combined with a vacuum system to draw the gas phase cigarette smoke inside sealed 250 ml flasks that contained medium number 4, 5, 6 respectively. 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. In 2, 5, and 6 media, the lymphocyte-containing PBS was supplemented with 30 %(v/v) saliva. After half the cigarette was used, the flasks were incubated for 20 minutes at 37 °C in a metabolic shaker, and then the procedure was repeated four times.

2.5. Survival of lymphocytes

The viability of the lymphocytes was measured at various time points (20 and 80 minutes) using a Trypan Blue exclusion test, both in the exposed and control medias.
The Joint Ethical Committee of Rafsanjan University approved the study plan. Means, standard deviations, and standard errors were computed. The analyses between six mediums were compared using Student's t

test or an ANOVA test.Statistical significance was accepted if $p < 0.05$. The statistical analysis was performed with an SPSS 13 package for Windows.

Results

The effects of cigarette smoke with/without saliva on the viability of PBL between 20 and 80 minutes were shown in figure 1. Comparison between viability of PBL at 20 minutes and 80 minutes showed that in the first 3 groups (PBS alone, PBS + whole saliva, PBS + stimulated saliva) there was no statically significant difference. However in next 3 media (PBS + cigarette smoke, PBS + cigarette smoke + whole saliva, PBS + cigarette smoke + stimulated saliva) the viability of PBL in comparison with the 20 & 80 minutes were shown to be statically different ($p<0.05$). Moreover, 80 minute exposure of the lymphocytes (in PBS) to cigarette smoke resulted in a time-dependent reduced survival of the cells.

Discussion

This study is another step to evaluate the lethargic synergic effect of cigarette smoke and saliva. The most surprising result in the 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 (10). Saliva secreted by the sub mandibular and sub lingual (Sm/Sl) glands was found to be highly cytotoxic compared with saliva secreted by the parotid gland.

The results showed that exposure of the lymphocytes suspension to cigarette smoke alone (without saliva), reduced survival rate of the cells in a time-dependent manner. Cigarette smoke-induced injury to lymphocytes has been previously described as anergy, various losses in cell function, genomic deletions, and other DNA injuries such as a decreased number of micronuclei or DNA adduct formation (11).In this respect, Yang et al. (12) discovered that exposure of human lymphocytes to water-soluble compounds, from cigarette smoke, resulted in DNA damage. The rapidly induced DNA damage was attributed to directly acting compounds from cigarette smoke. Traditionally, the effects of cigarette smoke were thought to be mediated by a direct attack of ROS and RNS on the surrounding biological macromolecules and cells.

Moreover, it has been demonstrated that cigarette smoke-borne aldehydes directly attack cells which is unrelated to the salivary effects on the cells (13). Furthermore, Yang and colleagues also reported that, acrolein (a highly prevalent aldehyde in cigarette smoke) induced DNA damage (12).

According to Hershkovich et al. study (9), 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 may be one mechanism that leads to the destruction of protein structures in the cellular membrane (14). 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 (15). The importance of saliva's antioxidant capacity was clearly

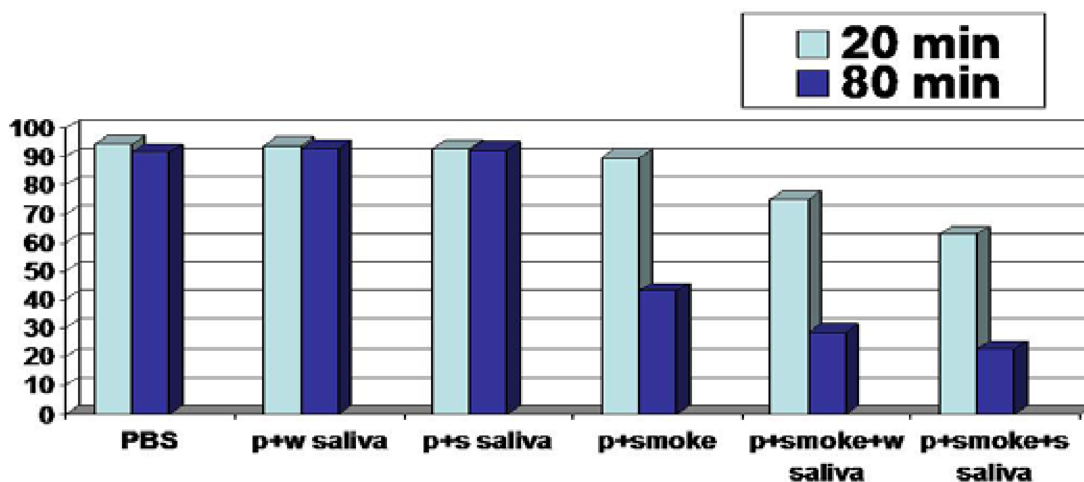

Fig. 1. Effects of cigarette smoke with/without saliva on viability of PBL in 20 and 80 min.

Table 1. The comparison between studies of viability of PBL

Intervention Study	Lymphocyte + Smoke		lymphocyte + smoke + Whole saliva		Lymphocyte + smoke + Stimulated saliva	
Time	20'	80'	20'	80'	20'	80'
Hershkovich, Nagler (2004)	-	52%	-	20%	-	-
Hansis, Nagler (2004)	-	56%	-	15%	-	-
This study (2007)	90%	43%	75%	29%	63%	23%

demonstrated by Nair et al. (16) who reported that saliva inhibited the production of radical oxygen species, the superoxide free radical and hydrogen peroxide (H2O2) 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, are a key player (17).

However, in the presence of cigarette smoke, saliva had the opposite effect in Hershkovich et al. and also in this study indeed it doubled the cigarette smoke-induced lethal effect. Within 80 minutes, over 70% of the lymphocytes were lost, both in our study and Hershkovich's study. They found that saliva in the presence of cigarette smoke dramatically enhanced the level of free radical production and, consequently, the lymphocyte killing rate. They concluded that the death rate of over 80% resulted from both the severe, lethal nature of the aldehydes and the metal-related reactive oxygen species (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 sub lethal only, leading to DNA damage, mutagenic transformation and the induction of cancer (12).

Hershkovich et al. presented a schematic which explained the mechanism of the process leading to oral cancer (9). It is based on both cigarette smoke-induced salivary-related and unrelated injurious factors, which act, either directly or indirectly to cause mutagenic aberrations (9).

These mechanisms are also supported by many studies demonstrating that the most widely used chemo preventive agents against oral pre-cancer lesions (in humans) and oral cancer (in vitro and in vivo models) are GSH and antioxidants (vitamin 5 A, E, b-carotene) and free radical scavengers (18).

To evaluate the lethal synergic effects of cigarette smoke along with whole or stimulated saliva, we exposed peripheral lymphocytes to cigarette smoke, alone and in the presence of whole or stimulated saliva. The current study demonstrates that in the 'wrong circumstances' saliva becomes highly deleterious, such as when it is exposed to CS.

It should also be noted that oral epithelial cells would be a better model to use than lymphocytes. However, these cells are technically much more difficult to use and are not as sensitive to oxidative stress. Accordingly, lymphocytes were chosen because they are known to be so sensitive to free radical-mediated attacks and thus are suitable for evaluating the CS effects as well.

The comparisons between Hershkovich et al. studies and this study are shown in table 1. Viability rate of PBL in the different studies at 80 minutes did not show any significant difference.

Smoking and drinking in many cases is a coincidental event. Many of smokers consume cigarette after eating. As a result, the evaluation of synergic effect of cigarette smoke and stimulated saliva as a pivotal player in the process leading to oral cancer, is crucial.

In this study, PBS was exposed to cigarette smoke in presence of stimulated saliva. Viability of PBL in this medium decreased significantly in comparison with control groups and even in presence of whole saliva. ($p<0.05$) Nevertheless, stimulated high flow rates drastically change percentage contributions from each gland, with the parotid contributing more than 50% of total salivary secretions. Moreover, stimulated saliva has more antioxidant agents (19).

It is demonstrated, the combined effects of tobacco and alcohol result in a synergistic effect on the development of oral cancer. The mechanism(s) by which alcohol and tobacco act synergistically may include dehydrating effects of alcohol on the mucosa, increasing mucosal permeability, and the effects of carcinogens contained in alcohol or tobacco. Secondary liver dysfunction and nutritional status also may play a role (20). On the other hand; this study suggested that increase of stimulated saliva can be another reason for synergic effect of tobacco and alcohol in development of oral cancer. Moreover, tobacco consumption after eating has more destructive effect in the oral cavity.

Conclusion

In summary, the results of this study showed that the cytotoxic effects of CS on PBL were significantly increased in the presence of saliva, especially stimulated saliva. Our data suggests saliva and especially stimulated saliva may be a pivotal player in the process leading to oral cancer.

References

1. Johnson N. Tobacco use and oral cancer: a global perspective. J Dent Educ. 2001;65:328-39.
2. Ko YC, Huang YL, Lee CH, Chen MJ, Lin LM, Tsai CC. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. J Oral Pathol Med. 1995;24:450-3.
3. Bloching M, Stephan D, Agha-Mir-Salim P, Berghaus A, Lautenschläger C, Grummt T. Ames test as biomarker. HNO. 2001;49:440-6.
4. Dayan D, Hirshberg A, Kaplan I, Rotem N, Bodner L. Experimental tongue cancer in desalivated rats. Oral Oncol. 1997;33:105-9.
5. Nishioka H, Nishi K, Kyokane K. Human saliva inactivates mutagenicity of carcinogens. Mutat Res. 1981;85:323-33.
6. Nagler RM, Klein I, Zarzhevsky N, Drigues N, Reznick AZ. Characterization of the differentiated antioxidant profile of human saliva. Free Radic Biol Med. 2002;32:268-77.
7. Nagler R, Lischinsky S, Diamond E, Drigues N, Klein I, Reznick AZ. Effect of cigarette smoke on salivary proteins and enzyme activities. Arch Biochem Biophys. 2000;379:229-36.
8. Nagler RM, Lischinsky S, Diamond E, Klein I, Reznick AZ. New insights into salivary lactate dehydrogenase of human subjects. J Lab Clin Med. 2001;137:363-9.
9. Hershkovich O, Oliva J, Nagler RM. Lethal synergistic effect of cigarette smoke and saliva in an in vitro model: does saliva have a role in the development of oral cancer?. Eur J Cancer. 2004;40:1760-7.
10. Carlsson J. Salivary peroxidase: an important part of our defense against oxygen toxicity. J Oral Pathol. 1987;16:412-6.
11. Finette BA, O'Neill JP, Vacek PM, Albertini RJ. Gene mutations with characteristic deletions in cord blood T lymphocytes associated with passive maternal exposure to tobacco smoke. Nat Med. 1998;4:1144-51.
12. Yang Q, Hergenhahn M, Weninger A, Bartsch H. Cigarette smoke induces direct DNA damage in the human B-lymphoid cell line Raji. Carcinogenesis. 1999;20:1769-75.
13. Friesen M, Maru G, Bussachini V, Bartsch H, Nair U, Nair J, et al. Formation of reactive oxygen species and of 8-hydroxy-2'-deoxyguanosine in DNA in vitro with betel-quid ingredients. IARC Sci Publ. 1988;89:417-21.
14. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids. 2003;25:207-18.
15. Nagler RM, Kitrossky N, Chevion M. Antioxidant activity of rat parotid saliva. Arch Otolaryngol Head Neck Surg. 1997;123:989-93.
16. Nair UJ, Floyd RA, Nair J, Bussachini V, Friesen M, Bartsch H. Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA in vitro with betel quid ingredients. Chem Biol Interact. 1987;63:157-69.
17. Schlotte V, Sevanian A, Hochstein P, Weithmann KU. Effect of uric acid and chemical analogues on oxidation of human low density lipoprotein in vitro. Free Radic Biol Med. 1998;25:839-47.
18. Kaugars GE, Silverman S Jr, Lovas JG, Thompson JS, Brandt RB, Singh VN. Use of antioxidant supplements in the treatment of human oral leukoplakia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1996;81:5-14.
19. Edgar WM. Saliva and dental health. Clinical implications of saliva: report of a consensus meeting. Br Dent J. 1990;169:96-8.
20. Boyle P, Zheng T, Macfarlane GJ, McGinn R, Maisonneuve P, LaVecchia C, et al. Recent advances in the etiology and epidemiology of head and neck cancer. Curr Opin Oncol. 1990;2:539-45.

ACKNOLEDEGEMENTS

The authors are grateful to Professor Rafael M. Nagler, Department of Oral and Maxillofacial Surgery, Oral Biochemistry Laboratory and Salivary Clinic, Rambam Medical Center and the Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel for his great advice relating to this research.