Evaluation of Antioxidant Capacity and Cotinine Levels of Saliva in Male Smokers and Non-smokers

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Abstract

Background: The cigarette compounds are associated with the increase in the incidence of oral cancer and precancerous lesions. Salivary antioxidant system plays an important role in anti-carcinogenic capacity of saliva. Cotinine, a nicotine metabolite, has a longer half-life in comparison with nicotine and is a suitable marker for exposure to cigarette smoke. This study aims to measure total antioxidant capacity (TAC) and cotinine level in saliva of smokers and non-smokers and compare salivary cotinine level and TAC in each group.

Methods: In this cross-sectional study, 32 smokers and 34 non-smokers were recruited by consecutive sampling from Department of Oral Medicine, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran. Salivary cotinine and TAC concentrations were determined using the enzyme-linked immunosorbent assay (ELISA) technique. For data analysis, correlation tests of Spearman, Mann-Whitney U, and independent samples t-test were used.

Findings: A significant difference was observed between the two groups in the mean cotinine level and in the mean TAC (P = 0.015, P = 0.027, respectively). TAC showed a weak negative correlation with the cotinine level, but the difference was not significant (P = 0.651).

Conclusion: Antioxidants are of great importance to smokers because antioxidants are able to scavenge free radicals found in cigarette smoke. According to the results of present study, the salivary TAC in smokers was lower than that of non-smokers, and the salivary cotinine level in smokers was higher than non-smokers. Therefore, smoking endangers the oral cavity health by reducing the salivary TAC. Further studies with a higher sample size and other factors affecting the salivary TAC are needed for definitive comment.

Keyword: Antioxidants; Cotinine; Saliva

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Introduction

Smoking is a harmful habit resulting in destructive effects on oral health and plays a pivotal role in occurrence of pre-cancer and cancer lesions. The smoke of cigarette contains several substances such as carbon monoxide (CO), nitrogen, nicotine, and free radicals like superoxide, hydroxyl, hydrogen peroxide, and reactive oxygen. Cigarette smoking can cause oral cancer through production of free radicals and oxidative damage.\textsuperscript{1,2} Free radicals in inhaled cigarette smoke damage cells by reacting with polyunsaturated fatty acids (PUFAs) in membranes of cells and nucleotides in deoxyribonucleic acid (DNA).\textsuperscript{3,5} It is well established that DNA damage is associated with the development of cancer.\textsuperscript{6,7} Therefore, free radicals can initiate and promote tumor progression and increase the probability of cancer incidence in various parts of the body like oral cavity.\textsuperscript{1,8}

Cotinine, a primary metabolite of nicotine, has a longer half-life in comparison with nicotine and is a suitable screening tool for smokers. Cotinine can be measured in body fluids such as plasma, saliva, and urine.\textsuperscript{9} Saliva is the first biologic fluid of the body which is exposed to cigarette smoke. Saliva, in addition to having lubricating properties, consists of various biochemical, anti-bacterial, and anti-oxidant substances. Therefore, it can be considered as the first line of defense against oxidative stress caused by free radicals.\textsuperscript{8,10} Salivary anti-oxidant system is believed to play a vital role in defensive mechanisms against oxidative stress. Reduction of antioxidants is one of the etiologic factors affecting the incidence of oral mucosal lesions.

Cigarette smoking is known to have potential impact on levels of salivary cotinine and salivary antioxidant system. Saliva is a first line of defense system against oxidative stress caused by cigarette smoke and its assessment is easy, helpful, and non-invasive. Therefore, more investigation on the salivary total antioxidant capacity (TAC) and salivary cotinine is needed. Hence, the aim of this study was to measure and compare salivary TAC and salivary cotinine levels in smokers and non-smokers and compare levels of salivary cotinine and TAC in each group to provide pilot evidence for the possible association between TAC and salivary cotinine.

Methods

In this cross-sectional study, 300 patients who were referred to Department of Oral Medicine, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran, from December 2018 to May 2019, were examined and after being assessed for inclusion and exclusion criteria, 66 individuals were enrolled in the study by consecutive sampling technique, which 34 of them were non-smoker and 32 individuals were smoker. This research was approved by the Institutional Local Ethics Committee of Mashhad University of Medical Sciences (registration number: IR.MUMS.DENTISTRY.REC.1397.053).

Inclusion criteria were as follows: personal consent for entering the study, men over the age of 18 years old, those who smoked cigarettes, smokers: not having smoked in the hour before saliva sampling, non-smokers: never smoked in their lifetime, absence of systemic illness, and no history of having systemic illness. The criteria for excluding patients from the study included the patients with systemic diseases,\textsuperscript{5,11,12} patients with any pathologic oral lesions, those who reported a history of drug abuse in the last three month (even supplements and vitamins),\textsuperscript{12} and those with periodontal pocket larger than three millimeters.\textsuperscript{13} First, all the patients who were eligible to enter the study were informed of the study protocol and objectives. In case of agreement, the consent form was fulfilled and signed by all of them. After obtaining the consent form and entrance of the participants to the study, a checklist including personal characteristics, use of cigarette or other tobacco, duration of smoking, etc. was completed for them. To collect saliva, unstimulated saliva was used. For research purposes, unstimulated saliva is preferred instead of the stimulated saliva, since stimulated saliva has low concentration of biomarkers and makes the diagnosis difficult.\textsuperscript{14} Unstimulated saliva in smoker and non-smoker participants was collected using spitting method.\textsuperscript{15} Participants were noticed to not eat or drink during two hours before the saliva collection. The smokers were forbidden to smoke cigarette one hour before saliva collection. To collect the whole unstimulated saliva, the patients were asked to gather saliva in their mouth and then pour it into a test tube. This was repeated for every 60 seconds for the duration of 5-15 minutes. By this method, approximately, five milliliters saliva was obtained. Saliva collection was performed in an upright position. Saliva was collected between 10 to 12 a.m. Samples were
centrifuged to remove squamous cells and cell debris for 15 minutes at 3000 g. To prevent the saliva proteins from degradation, the samples were stored at -80 °C until subsequent biochemical analyses. Then TAC and cotinine levels were measured using antioxidant and cotinine assay kits (ZellBio Co., Germany).

The measurement of TAC in saliva was done using ZellBio TAC assay kit in accordance with the manufacturer's protocol (ZellBio GmbH, Germany) to assay the antioxidant capacity based on the oxidation-reduction colorimetric assay at 490 nm wavelength. TAC level was considered as the antioxidant amount in the sample compared with the ascorbic acid which acts as the standard. This kit can determine TAC with 0.1 mM sensitivity (100 µmol/l). The intra- and inter-assay variation coefficient is specified to be < 3.4%. The salivary cotinine level was also measured using the cotinine kit (ZellBio GmbH, Germany) following the manufacturer's instructions. 40 microliter of the specimens, 10 µl of the cotinine antibody, 50 µl of the standards, and 50 µl of horseradish peroxidase (HRP)-streptavidin were pipetted into a well and incubated for 60 minutes at 37 °C. The wells were washed 5 times with 300 µl of diluted wash buffer. 50 microliter of chromogen reagents A and B was added to each well and incubated for 10 minutes at 37 °C. Next, 50 µl of stop solution was added to each well. The optical density (OD) of each well was determined at 450 nm within 10 minutes after adding the stop solution. Calculation of cotinine level was done according to the tables, diagrams, and standard curves provided by the manufacturer’s instructions.

All calculations were performed using the SPSS software (version 20, IBM Corporation, Armonk, NY, USA). For data analysis, correlation tests of Spearman, Mann-Whitney U, and independent samples t-test were used. The significance level was set at ≤ 0.05 for all tests.

Results

A total of 66 male patients in the age range of 18-62 years were selected from Department of Oral Medicine, School of Dentistry, Mashhad University of Medical Sciences. The mean age of the study participants was 35.76 ± 11.23 years. 32 individuals were smoker (48.5%) and 34 of them were non-smoker (51.5%). In this study, mean daily cigarette use was 7.2 ± 5.3 cigarettes. These two groups were assessed for variables of cotinine and TAC. In this study, the minimum and maximum values for TAC in non-smoker group were 0.20 and 4.99, and in smoker group were 0.01 and 0.67, respectively. The mean TAC in smokers was 0.63 ± 1.11, and in non-smokers was 0.17 ± 0.16. There was a significant difference between the mean values of TAC between these two groups (P = 0.027) (Table 1).

Table 1. Comparison of mean total antioxidant capacity (TAC) between the study groups

| Parameter | Groups | P     |
|-----------|--------|-------|
|           | Smokers (n = 32) | Non-smokers (n = 34) |
| TAC       | 0.17 ± 0.16       | 0.63 ± 1.11       | 0.027 |

Table 2. Comparison of mean cotinine between the study groups

| Parameter | Groups | P     |
|-----------|--------|-------|
|           | Smokers (n = 32) | Non-smokers (n = 34) |
| Cotinine  | 3.57 ± 2.77       | 2.06 ± 1.63       | 0.015 |

Table 3 showed that in smoker group, TAC was adversely correlated with cotinine; however, this correlation was weak, and this correlation in non-smokers’ TAC with cotinine was also adverse and weak, which means that along with the increase or decrease of cotinine, TAC was also slightly decreased or increased (Figure 1). However, this correlation between two variables in both groups was not significant.
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In smokers, the number of cigarettes smoked per day showed a weak positive correlation with TAC, but the value was not statistically significant (P = 0.396). In addition, there was a weak positive association between the number of cigarettes smoked per day and salivary cotinine level, but the correlation between these two variables was also not statistically significant (P = 0.101).

Furthermore, there was a weak positive correlation between smoking duration and TAC values in smokers. However, the correlation between these two variables was not statistically significant (P = 0.396). Smoking duration showed a weak positive correlation with salivary cotinine level, but the correlation between these two variables was not significant (P = 0.101).

**Discussion**

In this study, we assessed the salivary TAC and salivary cotinine level in smoker and non-smoker participants. The results of the study showed that there was a significant difference between the mean values of TAC and salivary cotinine level between these two groups. In addition, salivary cotinine level showed a weak negative correlation with TAC, but the value was not statistically significant.

In 2019, Sharma et al. showed consistent findings with our results in comparison of salivary cotinine level in active smokers, passive smokers, and non-smokers. In their study, salivary cotinine level in active smokers was significantly higher than non-smokers. In addition, Singh et al. measured the levels of salivary catalase, salivary α-amylase, and cotinine in smokers and non-smokers. In this study, in agreement with our study, the salivary cotinine level was significantly greater in smoker group. Recently, studies indicated that main metabolite of nicotine was cotinine. Half-life of nicotine is 30-150 minutes, whereas the half-life of cotinine is longer, about 20 hours. Due to longer half-life of cotinine, its accumulation in body is more stable and is used as a biological index for assessment of tobacco smoke exposure rate. Although our study showed a significant difference for the mean salivary cotinine level between case and control groups, the high level of cotinine in control group in comparison with control groups in other studies might be caused by air pollution in various regions of Mashhad, and also being exposed by second-hand smokers other than family and co-workers outside the home and workplace as well as consumption of foods containing nicotine, such as tomato, tea, and coffee, which could affect the cotinine level in control group.

Additionally, based on this study findings, the mean TAC in non-smokers was significantly higher than smokers. Therefore, cigarette use reduces the salivary TAC. These findings are in line with other studies. In the studies by Bakhtiari et al., Greabu et al., and Ahmadi-Motamayel et al., in line with our study, the salivary TAC in smokers was significantly lower than non-smokers. The study by Guenttsch et al. also showed that the TAC of saliva in smokers with periodontal diseases was lower in comparison with other groups. Moreover, the findings of several studies were inconsistent with ours. Zappacosta et al. and Buduneli et al. indicated that there was no difference in the TAC of saliva between smokers and non-smokers. Charalabopoulos et al. also showed that, despite increase in the level of plasma antioxidant in smokers, the TAC in saliva was not different between the two groups. It seems that these inconsistencies and discrepancies in the studies are due to different sample sizes, various methods for antioxidant measurement, and genetic and ethnicity diversities. It was reported that the imbalance in free radicals and antioxidant levels could damage cellular and extracellular constituents and play an important role in initiation and development of oral inflammatory diseases. Antioxidants confront with the harmful effects of free radicals and preserve the structure and integrity of the tissue. Antioxidants in saliva...
are able to scavenge free radicals in inhaled cigarette smoke before they injure cells.\textsuperscript{25,26} In both groups of this study, TAC showed a weak negative correlation with the cotinine level, indicating that along with an increase or a decrease in the cotinine level, TAC will slightly decrease or increase, respectively; however, this correlation between two variables was not significant. To justify this explanation, it can be stated that cigarette is not the only effective factor on TAC of saliva, and it is complicated to determine whether the difference in salivary TAC between smokers and non-smokers is due to the effects of cigarette use. Other factors including differences in antioxidant consumption from diets are also effective.\textsuperscript{27-29}

In the current study, the number of cigarettes smoked per day showed a weak positive correlation with TAC and also cotinine level in smokers, but this correlation was not statistically significant. To justify this explanation, it can be stated that the participants did not accurately respond to the questions related to the number of cigarettes smoked per day.

One of the limitations of this study was the lack of assessment of other effective factors on salivary TAC such as the diet. It is recommended to assess other effective factors on salivary TAC including diet in further studies. In addition, studies on larger sample sizes and passive smokers as a third group are warranted.

\section*{Conclusion}

The current study showed that the amount of salivary cotinine in smoker participants was higher than non-smokers, and also salivary TAC in smokers was lower than non-smokers. Therefore, cigarette smoking reduces the TAC of saliva and can compromise oral cavity health through this pathway. In addition, based on the findings of this study, TAC is weakly and adversely correlated with cotinine in smokers. To generalize the obtained findings, further studies considering other effective factors on salivary TAC are needed.

\section*{Conflict of Interests}

The authors have no conflict of interest.

\section*{Acknowledgements}

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\section*{Authors’ Contribution}

All authors designed and performed experiments, analyzed data and co-wrote the paper.

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ارزیابی ظرفیت آنتیاکسیدان و میزان کوتینین براق در مردان سیگاری و غیر سیگاری

چکیده

مقدمه: ترکیبات سیگار ارتباطی زیادی با آفتاب پر و سرطان دهان و ضایعات بین دهان دارد. سپس انتیاکسیدان براق، نقش مهمی در ظرفیت ضد سرطان براق ایفا می‌کند. کوتینین، منابع در طولانی‌ترین مدت با مشابهی با کوتینین دارد و نشانگر مناسبی برای قرار گرفتن در (TAC: Total antioxidant capacity) معرض گزارش‌های پزشکی به‌صورت حاضر با هدف اندوزه‌گیری ظرفیت تام انتیاکسیدان

کوتینین در براق مردان سیگاری و غیر سیگاری و همجنس، مقایسه سطح کوتینین و TAC بر روی دش از هر گروه انجام شد.

روش‌ها: در این مطالعه مقطعی، 22 گروه سیگاری و 23 گروه غیر سیگاری به روش نمونه‌گیری تصادفی، از بین بامیه‌های دهان، فک و صورت Enzyme-linked immunosorbent داشتند. دنده‌ای دندان‌پزشک دانشگاه علوم پزشکی مشهد انتخاب شدند. مقدار کوتینین و TAC به وسیله Independent t Mann-Whitney U Spearman انتخاب کردند. داده‌ها با استفاده از آزمون هیپوستاتی (ELISA) assay تجزیه و تحلیل قرار گرفت.

یافته‌ها: از نظر میانگین سطح کوتینین و میانگین TAC، بین دو گروه تفاوت معنی‌دار مشاهده گردید (به ترتیب P = 0 / 20 روند (P = 0 / 27 روند). هیپوستاتی ضعیف و معکوسی با سطح کوتینین داشت، اما این تفاوت معنی‌دار نبود (P = 0 / 25 روند).

نتیجه‌گیری: انتیاکسیدان براق در برابر سیگار اهمیت زیادی دارد. جدی می‌تواند با ارائه‌های آزاد موجود در دود سیگار مقابله کند.

واژگان کلیدی: آنتیاکسیدان، کوتینین، براق

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