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A comparative study of salivary nitric oxide between smokers and nonsmokers

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ABSTRACT

It has been shown that several oral diseases were associated with changes in nitric oxide concentration. Smoking may be involved in the pathogenesis of the diseases by affecting on salivary nitric oxide levels. The aim of this study was to compare the salivary nitric oxide levels between smokers and nonsmokers. In this case-control study, smokers as cases and age/gender-matched nonsmokers as controls were selected. Participants were asked to avoid eating, drinking, dental brushing or flossing for two hours before taking saliva specimens. Samples of unstimulated saliva were collected. Nitric oxide levels were measured by Griess reaction method. Data were analyzed by SPSS using Kolmogorov-Smirnov and Mann-Whitney tests, and multiple linear regression and Spearman correlation coefficient (P<0.05). Forty-four smokers, as cases, including 19 males (43.2%) and 25 females (56.8%) aged 19-25 years and 38 nonsmokers, as controls, including 21 males (55.3%) and 17 females (44.7%) aged 20-25 years were studied. There was no significant difference in salivary nitric oxide levels between smokers (range: 2-552; median: 66.8 microM) and nonsmokers (range: 0.1-857; median: 71 microM) (P=0.996). Multiple linear regression analysis showed that gender and age variables had no significant effect on salivary nitric oxide levels (P<0.05). In smokers, the salivary nitric oxide level presented an indirect significant correlation to smoking rate in term of pack-year (P<0.001; r= - 0.525). These findings suggest that salivary nitric oxide levels were reduced by an increase in rate of smoking.

Keyword: saliva, nitric oxide, smoking

INTRODUCTION

Saliva is an exocrine secretion containing various mixtures and can be used in diagnosing and evaluating a variety if systemic diseases as well as their complications. Nitric Oxide is one of the key factors of saliva. As it can affect the pathogenesis of the diseases, it has been of great importance as a diagnosis marker in recent years (1).

Nitric Oxide (NO) is formed as a result of the oxidation of guanidino nitrogen of L-arginine molecule. In nitric oxide synthesis a group of enzyme family (isoenzymes) referred to as nitric oxide synthases are effective: neuronal NOS (nNOS); Inducible NOS (iNOS); and endothelial NOS (eNOS).

NO has two biological functions: first, its origin can be vascular endothelial. Thus, it can function as a smooth muscle relaxant, platelet aggregation/adhesion inhibitor, and neurotransmitter. Second, in high volumes, it is formed by activated macrophages that function as a kind of cytotoxic molecule. It can affect the capability of macrophages in killing bacteria, viruses, protozoa, and tumoral cells (3). NO is an important regulator of numerous physiological,

defensive, inflammatory, and immune processes. Its pre-inflammatory effects including vasodilation, edema, cytotoxicity, and participating in cytokine-related processes may lead to tissue destruction (4). NO, also plays an important role in forming vasodilation as well as inhibiting vasoconstriction owing to the function of angiotensin II and endothelin (5). NO effects on cells participating in the process of inflammation have not been clearly identified and depend on cellular environment, NO concentration, and other probable factors (6).

The presence of excess NO can result in different kinds of RNS (Reactive Nitrogen Species) (8). NO can be transformed into different kinds of RNS including NO^+ , NO^-, $ONOO^-$. RNS and ROS (reactive oxygen species) are the body's most important free radicals. In oxidative stress conditions, the increase in ROS and RNS has destructive effects on cellular processes (9). RNS plays an important role in forming a destructive marker called 8-nitroguanine that can result in cellular death and injuries on tissues and organs through its destructive effects on proteins, DNA, and cellular lipids (3). The stress arising from ROS and RNS can have destructive and deadly effects on the cells. The relation existing among these factors with pathophysiological mechanisms has been shown in the incidence of a variety of diseases including atherosclerosis, neural degenerative diseases, and diabetes (10).

The changes in the concentration of Nitric Oxide in different oral areas are closely related to dental diseases. The measurement of saliva NO levels has been offered as a diagnosis criterion of periodontal diseases (11). A study done by Daghighet.al has indicated that NO generation increased in gingival fibroblasts as a result of simultaneous stimulation of TNF-alpha, IL-1beta, and IFN-gamma; NO generation increase has been suggested as an important factor in the pathogenesis of periodontal diseases (12). Bayindiret. al have reported higher levels of NI in the dental plaque of those suffering from dental caries (13). The increased levels of salivary NO in chronic and aggressive periodontitis (14), recurrent ulcerative (15), oral lichen planus (14), oral precancerous lesions (17), and dental caries have all been indicated and reported. In another study, the decrease of salivary NO in children suffering from dental caries (Rampant and early childhood caries) have been reported in comparison to those who do not suffer from dental caries (18). Moreover, having measured NO metabolites, Andrukhov et al found out that NO generation decreases in patients suffering from Periodontitis (19).

Other studies have shown the increase of a variety of oxidative stress factors in the saliva of smokers and the relation of these factors with aggravated dental diseases (20, 21). According to the findings of Marteus et al, smoking does not affect NO non-enzymatic generation of the salivary nitrite. However, smoking decreases NO enzymatic generation through its inhibitive effects on inducible NOS enzyme in squamous epithelium of oropharynx and bronchi (22). Another study done on laboratory animals indicated that NO metabolites decreased, to a great extent, after being exposed to smoking; the reason behind this decrease has been attributed to the decrease in eNOS enzyme gene expression (23). It has been shown that being exposed to smoking results in gastric ulcer healing delay through decreased gastric blood flow as well as reduced Angiogenesis in ulcer edges. cNOS reduced expression and enzyme activity have been suggested as the factors for the incidence of these ulcerogenic processes (24).

Based on the search made, few studies done concerning the effect of smoking on salivary NO level (25-29) have suggested contrary findings. Thus, the present study aims at evaluating the amount of salivary NO in smokers and non-smokers.

MATERIALS AND METHODS

In this case-control study, participants were selected from those referring to the Dentistry School of Kermanshah University of Medical Sciences. Sample size was measured through the following formula:

$$n = \frac{\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}\right)^2 \left({S_1}^2 + {S_2}^2\right)}{(\bar{X}_1 - \bar{X}_2)^2}$$

Having in mind α = 0.05 and power 1 - β = 90 % and according to the study done by Kurku et al (28) NO varied standard deviation in case and control groups were $S_1 = 170$ and $S_2 = 225$ respectively. The NO varied mean in case and control groups were $\bar{X}_1 = 210$ and $\bar{X}_2 = 407$ respectively. The minimum sample size obtained was 46 participants (23 for each group).

As many as 44 smokers as the cases and 38 age/gender-matched nonsmokers as controls were selected. Having taken their agreement and written consent salivary samples were collected.

Inclusion criteria of the present study:

- aged 18 to 25

- general health

Exclusion criteria of the present study:

- Taking any medicine in the last three months.
- Alcohol consumption.
- Dmft is equal to 3 or more (severe caries).
- Composite recovery [composite recoveries can affect salivary NO concentration (30)].
- Suffering from systemic diseases.
- Suffering from periodontitis diseases and any oral lesions.

For assessing the participants' general health as well as the health, their participants' medical as well as dental history were taken by the project executive. The participants underwent oral and dental examination to make sure that there are not any various dental caries, periodontal diseases, and composite recoveries.

In the present study, samples of unstimulated saliva were used. The participants were taught to avoid eating, drinking, dental brushing or flossing for two hours before taking saliva specimens. Each participant was asked to sit down calmly, and bend his or her head forward so that the saliva will be collected in the frontal area of mouth. Having swallowed the first saliva content, the recollected saliva was taken out of mouth and collected in the test tube (31).

Saliva samples were sent to the laboratory. Each sample was centrifuged at the speed of 3000 rpm for ten minutes and the upper part of the tube was used for evaluation. The samples obtained were kept in -80 centigrade degree until the laboratory study was done. NO concentration in samples of saliva was measured and registered using Griess reaction.

The data obtained was analyzed using SPSS18. Kolmogorov-Smirnov test indicates an abnormal distribution of salivary NO. Thus, the difference in the salivary NO level in case group and control group was evaluated using Mann-Whitney test. In order to study the effect of gender and age variables multiple linear regression was used. The relation between salivary NO level and smoking rate was evaluated by using Spearman correlation coefficient. In the present study the significant level of $P<0.05$ was reported.

Findings

In the case group 44 smokers, including 19 male smokers (%43.2) and 25 female smokers (%56.8) were studied. As for the control group, 38 non-smokers, including 21 male (%55.3) and 17 female (%44.7) were studied. With respect to gender distribution, there was no significant statistic difference in the two groups (P=0.275).

Kolmogorov-Smirnov test indicated that age (P=0.011) and salivary NO (P<0/001) had an abnormal distribution. However, the daily smoking rate (P=0.279), years of cigarette consumption (P=0.186), and pack-year (P=0.279) had normal distribution.

The age range of the smokers was 19-25 (median 24) and it was 20-25 for the non-smokers (median 23). According to Mann-Whitney test, the age difference existing between the two groups was not significant (P=0.772).

As for the smokers, the average consumption duration was 1.6±3.5 years, daily consumption was 4.8±9.3 cigarettes, and the pack-year was equal to 1.2±1.8 (table 1).

Table 1. Demographic features of study groups

	Control group (non-smokers)	Case group (smokers)
Number of participants	38	44
gender: Male(%) Female(%)	 (55.3%) 21 (44.7%) 17	 (43.2%) 19 (56.8%) 25
Age group (year) ≤21 22 – 23 24 - 25	 (7.9%) 3 (47.4%) 18 (44.7%) 17	 (13.6%) 6 (34.1%) 15 (52.3%) 23
Cigarette consumption: Smoking duration (year) Daily consumption Pack.year	 - - -	 3.5± 1.6 9.3± 4.8 1.8 ± 1.2

Consumption

According to Mann-Whitney test, the difference between smokers and non-smokers was not significant with respect to salivary NO level (P=0.996) (table 2). In order to study this difference in the adjusted state with age and gender variables multiple linear regression was used, and logarithm transformation was used for normalizing the salivary NO level. Regression analysis indicates that none of these variables has a significant effect on salivary NO level (table 3).

Table 2. The comparison of salivary NO in case and control groups

group	number	Nitric Oxide (micromole)				Z	P value*
		Interquartile range	Median	Mean±SD	range		
Case group	44	95.2	66.8	106.2 ± 130.4	2-552	-0.005	0.996
Control group	38	104.9	71	199.4±138.2	0.1-857		

*testMann-Whitney **

Table3. The findings of the study of the effects of age, gender, and cigarette consumption on salivary NO level using multiple linear regression

	β coefficient	Standard deviation	Standardized β coefficient	t statistic	P value
Regression fix	4.527	2.740		1.649	0.103
Age	-0.017	0.117	-0.017	-0.145	0.885
Female	0	-	-	-	-
Male	-0.190	0.361	-0.061	-0.527	0.600
Cigarette consumption (non-smokers)	0	-	-	-	-
smokers	-0.163	-0.452	-0.052	-0.452	0.653

Spearman correlation coefficient suggested that in smokers, the salivary NO level had a significant indirect relation with consumption rate on the pack-year basis (P<0.001, r= -0.525) (diagram 1).

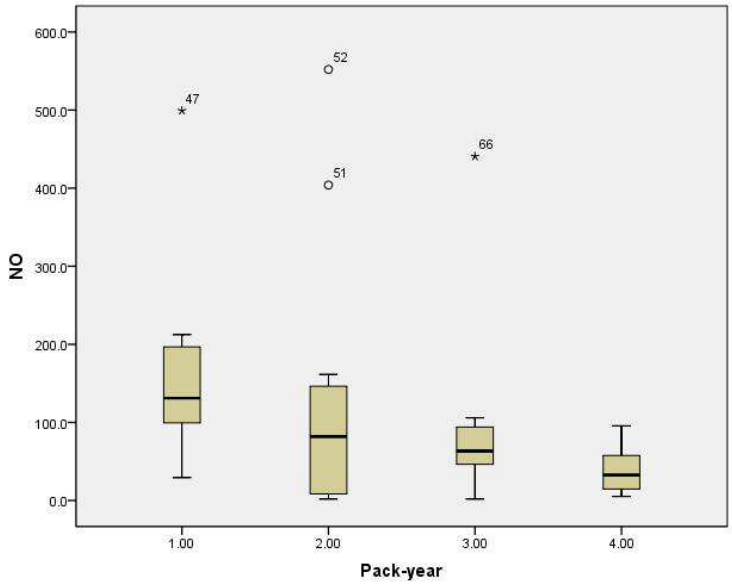

Diagram 1. Box plot of salivary NO level base pack-year cigarette

DISCUSSION

Smoking can bring about some changes in the individual's salivary system. The level of saliva in smokers decrease considerably with the increase of smoking duration and ageing.

With respect to quality, smokers have a more concentrated saliva than non-smokers (32). Moreover, in smokers amylase enzymes, lactic dehydrogenase, and acid phosphatase all decrease and the function of the salivary immunity system is disrupted (33, 34). In smokers salivary EGF (Epidermal growth factor) shows lower levels (35). According to the study done by Abdolsamadi et al, smoking is closely related to the remarkably decreased levels of superoxide dismutase antioxidants, glutathione peroxidase, and peroxidase (36).

Evaluating NO as one of the salivary mixtures and identifying the factors effective on its concentration is of great importance for NO is closely related to some critical states including malign lesions, oral precancerous lesions (37) and periodontitis (38) and also as a defensive mechanism against bacteria generation of dental plaque (26). Thus, in the present study, the effects of smoking on NO level was evaluated using a case-control method.

The present study indicated that although the difference in salivary NO level was not significant between smokers and non-smokers, the aforementioned level was lower among the smokers. The lack of significant statistical difference can be justified by low consumption of cigarette in the smoking group(average consumption duration was 1.6±3.5 years, daily consumption was 4.8±9.3 cigarettes, and the pack-year was equal to 1.2±1.8); theconsumption level in the smoking group was not that high to bring about significant difference in comparison to non-smokers.In confirming this state, Spearman correlation coefficient suggested that the salivary NO level decreases significantly by cigarette consumption increase. It seems that by cigarette consumption increase in smokers, on the basis of pack-year, considerable NO decrease and creating a significant difference (in comparison to non-smokers) is likely which calls for further studies. Besides, regression analysis indicated that age and gender variable did not have a significant effect on salivary NO level and thus one can attribute the tendency towards increased NO to cigarette consumption in smokers. In confirming the decreasing effect of smoking on salivary NO, the studies done by Bodis and Haregewoin (25) and Carossa et al have all indicated that salivary NO level in smokers was remarkably lower than that of the non-smokers. Marteus et al have attributed the decrease salivary NO level in smokers to inhibitive effects of smoking on inducible NOS enzyme existing in squamous epithelium of oropharynx and thus decreased NO generation (22).

Contrary to the present study, Wadhwaet a have noticed that smoking was closely related to increased salivary NO level in individuals suffering from chronic periodontitis and the have reported the minimum salivary NO concentration in non-smokers (27). Also, Kurka et al have observed that NO level in smokers was considerably higher than that of the non-smokers. NO level increased remarkably in smokers after smoking (28). Moreover, Preethi et al have found out that consuming cigarette and chewing tobacco was closely related to salivary NO increase (29). The contrariness in the findings of various studies can be attributed to different measurement methods as well as different case groups with respect to effective illnesses on salivary NO.

CONCLUSION

The present study indicated that smoking can decrease, dose relatedly, the salivary NO. Age and gender, as accompanying variables, did not show remarkable effects on the changes in salivary NO level.

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