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ORIGINAL ARTICLE

Status of Salivary Nitric Oxide Levels and Buccal Epithelial Cell DNA Damage in Potentially Malignant Disorders – A Biochemical Study

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ABSTRACT

Oral cancer is a progressive, multistage disease in which changes in genetic structure and cellular morphology occur from the normal to the premalignant state and then to the malignant state. Nitric oxide ($NO^{\cdot}$) is an uncharged molecule with an unpaired electron. It is highly reactive and interacts with DNA molecules, resulting in DNA damage. **Objective:** To evaluate the salivary nitric oxide levels and buccal epithelial cell DNA damage in patients with potentially malignant oral disorders. **Methods:** The salivary nitric oxide levels and buccal epithelial cell DNA damage were estimated in 20 healthy individuals without oral lesions, in 20 subjects having smoking and/or tobacco chewing habits without oral lesions, and 20 patients with a potentially malignant oral disorder. **Results:** The salivary nitric oxide levels were significantly greater in the subjects with tobacco chewing and/or smoking habits without oral lesions than in the healthy controls. Similarly, the extent of DNA damage was higher in the subjects with potentially malignant disorders and in the subjects with tobacco chewing and/or smoking habits without oral lesions than in the healthy controls. **Conclusion:** The encouraging results of the present study indicated the potential involvement of nitric oxide in the pathogenesis of potentially malignant oral disorders.

Keywords: *saliva, nitric oxide, comet assay, potentially malignant disorders*

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INTRODUCTION

Cancer causes about 20% of all deaths in developed countries and 10% of all deaths in under-developed countries worldwide. It is estimated that around 43% of mortalities due to malignancies are caused by harmful diet, sedentary or inactive lifestyles, infection, alcohol consumption, and tobacco use.[1] The simultaneous usage of tobacco and alcohol is an additive component that causes malignancies of the pharynx, larynx, esophagus, and oral cavity.[1] Oral malignancies have a widely variable rate of occurrence, and they have been reported to constitute around 12% of all incidences of malignancies in men and 8% of all malignancies in women. It has been estimated that in India, 83,000 new cases of oral malignancy occur every year.[2]

Most oral squamous cell carcinomas are believed to develop from potentially malignant oral disorders. The identification of high-risk potentially malignant oral disorders and timely intervention at various stages could constitute a means of reducing morbidity, mortality, and the cost of treatment associated with squamous cell carcinoma.[3] Nitric oxide (NO) is found in normal and cancerous tissues, but the levels are higher in cancerous tissues than in normal tissues.[4,5] Nitric oxide in the tissues is produced by several nitric oxide synthases (NOS), including inducible NOS (iNOS). The overexpression of iNOS mRNA has been established in potentially malignant disorders.[6] The expression of iNOS has also been identified in squamous cell carcinoma (SCC) of the head and neck region by using non-quantitative gene expression techniques and immunohistochemistry.[7]

Nitric oxide facilitates DNA damage through the production of reactive nitrogen species (RNS), the formation of carcinogenic nitrosamines, and the inhibition of the DNA repair mechanism. Hence, nitric oxide can be considered a tumor initiator. As an initiating agent, nitric oxide promotes the various phases of cancer development, the effects of which range from cellular and molecular alterations to the development of cancerous lesions and the regulation of various stages of tumor biology.[8] Hence, the purpose of this study was to evaluate the levels of nitric oxide in saliva and the DNA damage to buccal epithelial cells in healthy individuals and in subjects with potentially malignant oral disorders. Most previous studies have focused only on malignant disorders, potentially malignant disorders. In addition, previous studies have concentrated only on serum, not saliva. Our study examined potentially malignant disorders using saliva samples. In addition, we correlated the levels of salivary nitric oxide with the extent of DNA damage in the study subjects.

METHODS

A case control study was conducted with subjects who reported to the Department of Oral Medicine and Radiology. After obtaining the institution's ethical approval, the nature and purpose of the study were explained, and informed written consent was acquired from the subjects who were included in the study. A detailed case history of each subject was recorded, and all subjects included in the study underwent a thorough oral examination.

The study consisted of three groups of 20 subjects between the ages of 20 and 60 years. The groups were comprised of (1) Control Group (C): Twenty healthy subjects without any systemic or oral diseases; (2) Study Group 1 (S1): Twenty subjects with smoking and/or tobacco chewing habits without any oral lesions; and (3) Study Group 2 (S2): Twenty subjects having smoking and/or tobacco chewing habits and diagnosed with potentially malignant oral disorders both clinically and histopathologically.

Strict inclusion and exclusion criteria were followed. Group C included subjects with no history of oral or systemic diseases, no long-term medication, and no history of substance abuse. Group S1 included subjects with one or more of the following habits: smoking, chewing tobacco or betel nut, or alcohol consumption with no oral lesions. Group S2 included subjects with one or more of the habits of smoking, chewing tobacco or betel nut, or alcohol consumption and who were diagnosed histopathologically with potentially malignant oral disorders such as oral submucous fibrosis, erosive lichen planus, and leukoplakia. Individuals with a history of recent infection, subjects with systemic illness, and subjects on any medication were omitted from the study.

Saliva samples

Unstimulated whole saliva was collected under resting conditions 1 hour after food intake between 8:00 a.m. and noon. The patients were instructed to accumulate saliva in their mouth and spit it into a test tube for a period of 10 minutes. After the collection procedure, the saliva samples were centrifuged at 800 rpm at 48 °C for 10 minutes. The resultant supernatant was later subjected to biochemical analysis.

Buccal epithelial cells

After instructing the subjects to rinse their mouths with water, scrapings were taken from the buccal mucosa using a slightly moistened metal spatula. The collected cells were immediately smeared on pre-cleaned microscopic slides, which were later used in the comet assay.

Griess method for salivary nitric oxide estimation[9]

Principle

The nitric oxide levels were determined based on the conversion of nitrate to nitrite with the help of an enzyme using the colorimetric detection of the nitrites. In this diazotization reaction, acetified nitric oxide produced a nitrosating agent. The nitrosating agent reacted with sulfanilic acid, resulting in the production of the diazonium ion, which was then coupled with N-(1-naphthyl) ethylenediamine. This procedure resulted in the formation of chromophoricazo derivatives, which absorbed light at 540–570nanometers. The reagents used were a sulphanilamide solution and an N-(1-naphthyl)-ethylene diamine (NED) dihydrochloride solution.

Procedure

The reagents were prepared prior to the estimation of the nitric oxide. The sulphanilamide solution was prepared by adding 0.5g of sulphanilamide to 100ml of 20%v/v Hcl. The N (1-naphthyl)-ethylene diaminedihydrochloride solution was prepared by adding 0.3g of solid reagent (NED dihydrochloride) to 100ml of 1%v/v Hcl.

A standard nitric oxide solution was pipetted into five different test tubes ranging from 0.2–1ml. The volume of each test tube was increased to 1ml by adding distilled water: 1ml of the distilled water was placed in a separate test tube, which served as a blank; 1ml of the sulphanilamide solution and 1ml of the NED dihydrochloride solution were added to each test tube and mixed. All the test tubes were then incubated at room temperature for 10minutes. The absorbance was subsequently measured at 550nm.

From each saliva sample, 100µl was placed in a test tube. The volume was prepared up to 1 ml with 0.9ml

Table 1. Gender and age distribution of the subjects of the three groups

Groups	Gender		Age				
	F	M	Mean±SD	Age Group			
				21-30	31-40	41-50	51-60
C	8 (40)	12(60)	30.2±10.035	12 (60)	4 (20)	3 (15)	1 (5)
S1	5 (25)	15(75)	39.65±15.397	9 (45)	1 (5)	1 (15)	9 (45)
S2	4(20)	16(80)	40.85±14.543	9 (45)	1 (5)	1 (5)	9 (45)

*C= control group healty subjects, S1= study group 1:subjects with smoking and/or tobacco chewing habits without any oral lesions, S2 = study group 2 subjects with smoking and/or tobacco chewing habits and diagnosed with potentially malignant oral disorders both clinically and histopathologically, F= females: M=males, SD=standard deviation.

of distilled water. Then 2ml of sulphanilamide solution was added and kept for five minutes, after which 2ml of the NED dihydrochloride solution was added. After 10 minutes, the absorbance was measured at 550nm. The concentration was calculated using a calibration plot that was prepared from a series of standard nitrites. The nitric oxide levels were measured in µM/l.

Comet assay for the detection of DNA damage[10]
Encapsulation
A molten low-melting-point agarose was used to suspend the cell samples at 37°C. A microscopic slide was used to cast the mono-suspension. A cover slip made of glass was held at an angle to the microscopic slide. The mono-suspension was applied at the contact point between the coverslip and the slide. The agarose in its molten state was spread to form a layer as the coverslip was lowered onto the slide. The agarose was gelled at 4°C after which the coverslip was detached. The agarose formed a matrix of carbohydrate fibers that encapsulated the cells, securing them in place.

Lysis
The slides were immersed in a solution that caused the cells to lyse. The solution used for lysis in the comet assay was composed of a detergent (Triton X-100), and highly concentrated aqueous salt. Depending on the type of damage, the pH of the solution used for the lysis was adjusted between neutral and alkaline pH.

Electrophoresis, imaging, and analysis software
After the cells were lysed, the slides were washed in distilled water for 1–2 hours at 4°C to eliminate all salts and then dipped in an electrophoretic solution. The solution was then adjusted for pH based on the type of DNA damage that was being studied. The slides were left for approximately 20 mins in the electrophoretic solution after which an electric field was applied. The double helix of the DNA was denatured to a single-strand nucleoid.

An electric field of one volt per centimeter was applied for approximately 20mins. At the pH of 7, the slides were neutralized and stained with a fluorescent that was DNA-specific. It was then evaluated using a microscope attached to a charge-coupled device, which was then connected to a computer for image analysis.

Comet Score software.
The data were then analyzed using SPSS version 17 software. An ANOVA test was used to compare the groups. A chi-square test was used to compare the association between age and gender in all three groups. Pearson's correlation was used to correlate the levels of nitric oxide with the DNA damage in all three sample groups.

RESULTS

The analysis of the demographic data on the control group (C), the study group (S1), and the study group (S2) (Table 1). Comparative analysis of mean salivary nitric oxide levels between the healthy controls (C)), subjects with smoking and/or tobacco chewing habits with no oral lesions (S1), and subjects with potentially malignant oral disorders (S2) (Table 2). Analysis of mean buccal epithelial cell DNA damage in the healthy controls (C), subjects having smoking/ tobacco chewing habits without any oral lesions (S1), and subjects with potentially malignant oral disorders (S2) (Table 3).

Analysis to determine the statistical significance of salivary nitric oxide
The comparison of nitric oxide levels in the saliva samples drawn from the C group and the S1 group showed highly statistically significant results ($p<0.001$). Similarly, the comparison of nitric oxide levels in the saliva samples drawn from the C group and the S2 group showed highly statistically significant results. (Table 2).

The comparison of the extent of DNA damage in the C group with the extent of DNA damage in the S1 group showed a highly statistically significant difference ($p <0.001$). Similarly, the comparison of the DNA damage the C group and the S2 group showed a highly significant difference ($p<0.001$). (Table 3).

Table 2. The salivary nitric oxide level of the subjects in all groups

Groups	Salivary Nitric Oxide level (µM/l)				
	N	Mean±SD	Minimal	Maximum	p*
C	20	76.11±2.33	70.48	81.18	<0.001
S1	20	90.09±2.53	84.95	96.18	<0.001
S2	20	105.21±2.68	99.17	111.37	<0.001

C= control group healty subjects, S1= study group 1:subjects with smoking and/or tobacco chewing habits without any oral lesions, S2 = study group 2 subjects with smoking and/or tobacco chewing habits and diagnosed with potentially malignant oral disorders both clinically and histopathologically, SD=standard deviation, *ANOVA

Table 3. The DNA damage measurement of the subjets in all groups

Groups	N	Mean±SD	Minimal	Maximum	p*
C	20	9.15±0.9132	7.15	11.15	<0.001
S1	20	15.748±1.0095	13.74	17.65	<0.001
S2	20	21.1565±1.0476	19.23	23.79	<0.001

C = control group healty subjects, S1= study group 1:subjects with smoking and/or tobacco chewing habits without any oral lesions, S2 = study group 2 subjects with smoking and/or tobacco chewing habits and diagnosed with potentitally malignant oral disorders both clinically and histopathologically, SD=standard deviation, * ANOVA.

Correlations of salivary nitric oxide levels and extent of DNA damage

The results showed that the correlation between nitric oxide levels and the extent of DNA damage in the C group and the S2 group was statistically significant and moderately positive. The results showed a strong, highly statistically significant correlation between nitric oxide levels and the extent of DNA damage in the S1 group (Figure 1).

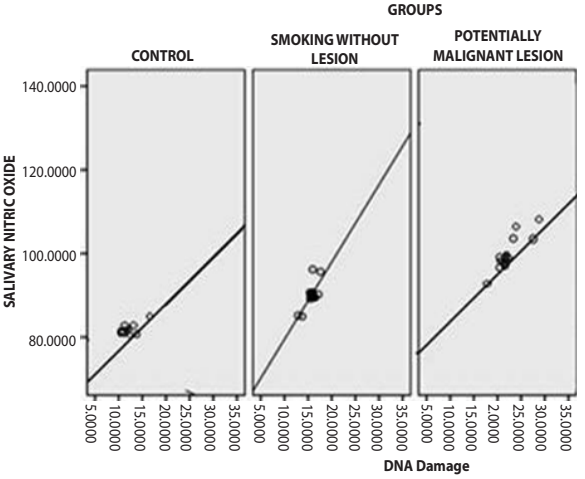

Figure 1. Correlations of DNA damage with nitric oxide levels among groups. The results showed a strong, highly statistically significant correlation between nitric oxide levels and the extent of DNA damage in the S1 group (smoking without lesion).

DISCUSSION

ROS and other metabolites, such as nitric oxide (NO), hydroxyl radicals (OH), malondialdehyde, super oxide anions (O_2), and hydrogen peroxide (H_2O_2), are involved in the process of cancer formation. Free radicals act as initiators and/or promoters of oral cancer.[11] Patel et al. assessed the mean plasma level of nitric oxide in healthy subjects without tobacco habits, individuals with tobacco habits, patients with potentially malignant oral disorders, and patients with oral malignancy. The results showed an increased mean of plasma nitric oxide levels in patients with potentially malignant oral disorders and oral malignancy.[12]

In the present study, we investigated salivary nitric oxide levels instead of serum. The salivary levels were found to be statistically significantly increased. Based on these results, saliva could be used effectively to measure nitrative stress levels in tobacco users and in potentially malignant oral disorders.

The mechanism of the increase in the nitrative stress levels in the saliva and serum is attributed to the dietary intake of nitric oxide from tobacco and its related products. Nitric oxide is absorbed from the upper gastrointestinal tract and is actively transported by the salivary glands from the plasma to the saliva.[13] Nitric oxide, which is either cytotoxic or cytostatic, interacts with a many molecular targets. The overexpression of nitric oxide synthase in chronic inflammation leads to genotoxicity. Nitric oxide mediates DNA damage through the formation and generation of reactive nitrogen species, carcinogenic nitrosamines, and the inhibition of mechanisms of DNA damage and repair. Therefore, it can be considered an agent that initiates tumor formation.[14]

Assessments of the risk of cancer could be evaluated by methods such as those that measure mutator genes and epigenetic changes and identify the mutation or deletion of oncogenes and anti-oncogenes. However, these techniques are both time consuming and costly. Hence, the present study was conducted to measure DNA damage using the comet assay in suspected cases in order to identify high-risk subjects. The comet assay is a sensitive technique that is known to estimate DNA damage and provide adequate information about individual cells. It is also less time consuming than other techniques.[15] Our study found a significant increase in the DNA damage in the buccal epithelial cells, which was estimated by comet assay using the CometScore software to analyze the samples drawn from the subjects in three groups, which was

in accordance with previous studies conducted by Manikantan et al., Mukherjee et al., and Jyoti et al.[16-18] The present study also aimed to evaluate DNA damage in relation to salivary nitric oxide levels in healthy controls, subjects with smoking and/or tobacco chewing habits, and subjects with potentially malignant oral disorders. The results showed a significant positive correlation between salivary nitric oxide levels and the extent of DNA damage in each group. It was observed that the increase in the levels of salivary nitric oxide appeared to have a marked effect on the extent of DNA damage. Because the alterations in salivary nitric oxide levels and the extent of DNA damage were the most prominent in the subjects with smoking and/or tobacco chewing habits, it can therefore be concluded that lifestyle plays a central role in the onset of potentially malignant oral disorders. In addition, the results revealed that the increase in salivary nitric oxide levels and the extent of DNA damage were higher in patients with potentially malignant oral disorders among the three groups. This result indicates that increased salivary nitric oxide levels affect the extent of DNA damage, which is one of the causative factors for potentially malignant oral disorders. The results of the present study shed light on the source and the role of nitric oxide in the pathogenesis of potentially malignant disorders by causing DNA damage. Therefore, saliva could be used as a diagnostic marker for the early detection of oral cancer. In the present study, we used saliva samples for the assessment of nitric oxide levels and exfoliative cytology for the collection of epithelial cells from buccal mucosa for DNA examination. Both methods of sample collection are inexpensive, facile, and non-invasive.[19-21] Hence, salivary nitric oxide and the comet assay of buccal cells optimally met all of the above requirements.[22]

CONCLUSION

The promising results of our study indicate the possible involvement of nitric oxide in the pathogenesis of potentially malignant oral disorders. In addition to the increased nitric oxide levels in potentially malignant disorders, the comet assay could be considered an appropriate and reliable test for assessing DNA damage. The present study also demonstrated the genotoxic effects of tobacco and its related products. The results of our study suggest the importance of the assessment of salivary nitric oxide and buccal epithelial cell DNA damage in the diagnosis of potentially malignant disorders. Therefore, this assessment could be used as as molecular marker for early diagnosis. Future studies need to be conducted using a larger sample size, including patients who are diagnosed with oral cancer, in order to confirm the role of DNA damage and nitric oxide levels in the pathogenesis of carcinogenesis.

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