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Original Research

Estimation of Salivary and Tissue Nitric Oxide Levels in Oral Squamous Cell Carcinoma: A Biochemical Study

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ABSTRACT

Objective: The study was conducted to estimate the salivary and tissue nitric oxide (NO) levels in healthy individuals and subjects with squamous cell carcinoma of the oral cavity.

Methods: In this study, the salivary and tissue NO levels were estimated in 20 healthy subjects and 20 patients with oral squamous cell carcinoma (OSCC).

Results: The mean salivary NO levels in Group I (control group) was 78.59 µM/L (standard deviation=5.91608), while the mean salivary NO levels of Group II (study group) were 115.6765 µM/L (standard deviation=0.9431). The mean tissue NO levels in Group I (control group) was 87.6315 µM/L (standard deviation=1.91631), while the mean tissue NO of Group II (study group) was 172.376 µM/L (standard deviation=0.84774.

Conclusion: Our results illustrated that the increase in the NO levels in the saliva is positively correlated with the NO level in tissues; hence, salivary NO level can be used as a potential diagnostic biomarker in OSCC.

Keywords: Oral cancer, nitric oxide, malignancy, neoplasm

INTRODUCTION

Oral cancer accounts for 2%–4% of all cancer cases across the world. About 90% of all oral neoplasm cases are estimated to be oral squamous cell carcinoma (OSCC) (1). It is categorized as the 12th most frequent cancer worldwide (2). The incidence rate of oral cancer is highest in the Southeast Asian countries and in India. In addition, 90%–95% of all cancers of the oral cavity are OSCC in India (3). The prevalence of cancer has been estimated to rise from 1 million in 2012 to >1.7 million in 2035 by the International Agency for Research on Cancer. This prediction implicates that the mortality due to cancer will also rise from 680000 to 1–2 million during the same period (4).

Cancer deaths mainly occur due to the consumption of tobacco and/or alcohol, sedentary lifestyles unhealthy diet, and infection (5). The overall 5-year survival rate has been evaluated to be approximately 50% (6), and more than half of the cases of OSCC are diagnosed at a later stage (7).

In the initial periods of this disease, the patients are usually asymptomatic; hence, the identification, discovery, and diagnosis of this disease become tough (8). Timely and accurate clinical diagnosis as well as decision-making for treatment plan along with adjuvant therapy are important because of the chances of secondary metastasis and the high recurrence rate (9). The diagnosis is established mainly on extensive clinical and histopathological exploration of the suspicious lesion, but it may remain unnoticed at certain hidden sites (10). Hence, the evolution of non-invasive, reliable, safe, and advantageous diagnostic markers is currently needed.

Biomarker is termed as any structure, substance or process that can be estimated in the body or its products that impacts or predicts the incidence of disease or the outcome (11). Tumor markers are substances that are specific for certain tumors or cancer cells and hence are important for diagnostic and prognostic purposes in oral cancer patients (12).

NO plays a role in the pathological process of cancer (13). It is the outcome of the transformation of L-arginine to L-citrulline and is catalyzed by the nitric oxide (NO) synthase (NOS) enzyme. The components essential for this reaction are oxygen and other cofactors, including heme, flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin (14).

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It has been suggested that several types of cancer-related events are regulated by NO (15). It plays a role in certain genotoxic events and causes several types of DNA damage during the initial stages of carcinogenesis (16). Three isoforms of NO synthase have been demonstrated so far, 2 of which are constitutive NOS (cNOS) and the third form is inducible (iNOS) by cytokines and endotoxins. The generation of reactive nitrogen species (RNS) occurs through a reaction of NO with either oxygen or other free radicals that exert numerous biological effects (17). NO could be either cytotoxic or cytostatic and interacts with several molecular targets present within the cell structure. Varying reactions to NO have been demonstrated in cells within various tissues**.** In chronic inflammation, the overexpression of the NOS leads to genotoxicity. NO causes damage of DNA by manufacturing RNS, generating carcinogenic nitrosamines, as well as inhibiting the mechanism responsible for DNA damage restoration. Hence, NO can be considered as a tumor-initiating factor (13). It also influences other stages of the process of cancer formation. The stretch of effects of NO is quite broad in tumor genesis, which includes its participation in the transformation of the cells, genesis of the neoplastic lesions, and initiation and regulatory mechanisms of the metastatic events (18).

Hence, the current study was planned to evaluate the significance of NO levels as an adjuvant diagnostic marker in OSCC by estimating the salivary and tissue NO levels in the squamous cell carcinoma of the oral cavity.

The objectives of the present study were to assess and compare the salivary NO and the tissue NO levels in healthy individuals and subjects with OSCC. The current study also co-related the levels of NO in the tissue and saliva of healthy and study subjects.

METHODS

The present study was a case-control study conducted on subjects reporting to the Department of Oral Medicine and Radiology. A total 40 patients were included in the study, and the study sample were divided into the following groups:

Control Group (Group I): 20 healthy subjects without any systemic and oral diseases (normal).

Study Group (Group II): 20 subjects diagnosed clinically and confirmed histopathologically with OSCC.

The subjects selected were of age 20–70 years in both the groups. The inclusion criteria of Group I comprised of healthy individuals with no history of oral or systemic diseases, no oral adverse habits such as consumption of tobacco (smoking)/betel nut and alcohol, and no use of any medication. The Group II included subjects who were clinically diagnosed and histopathologically confirmed with OSCC and those with adverse oral habits such as consumption of tobacco (smoking)/betel nut and alcohol. The exclusion criteria comprised of subjects with any systemic diseases, those diagnosed with malignancies at sites other than the oral cavity, those on any long-term medication, those with any other oral mucosal lesions including potentially malignant disorders (PMDs), and those with OSCC without any history of adverse oral habits.

Main Points:

- The current study assessed and compared the salivary NO and the tissue NO levels in healthy individuals and in subjects with OSCC and a positive correlation was obtained.
- The results of the study suggests that salivary nitric oxide can be used as a potential diagnostic marker in OSCC.
- Saliva provides a non-invasive, reliable, cost effective and safe method for screening a large population.

Method of Data Collection:

Collection of samples

Institutional ethical clearance was acquired before the commencement of the study. The goals of the study were described, and informed consent was obtained from individuals involved in the study. Comprehensive case history was documented along with complete inspection of the oral cavity for all subjects.

Collection of saliva

Unstimulated saliva was obtained from the subjects by using the "Spit Technique." Subjects were advised not to eat, drink, or smoke an hour prior to sample collection. They were seated on the dental chair and asked to tilt their head forward. They were then asked to not speak or swallow any saliva. They were also told to expectorate into a sterile graduated container for 8–10 min. The salivary sample collected represented the fluid contents of the whole mouth. The sample obtained was later centrifuged at 3000 rpm for 10 min, and the supernatant was collected and stored at −20°C.

Collection of the tissue

For tissue specimen, a segment of the oral mucosa was resected from the OSCC site of the patients through biopsy.

The tissue samples for the control group were acquired during operculectomy or frenectomy (only non-inflamed tissues were considered).

Fresh tissue samples obtained during surgical intervention were properly cleansed to remove any blood stains or any other necrosed tissues with 0.9% HCl. The samples were later dried on blotting paper and weighed. One gram of the sample was homogenized in 10 mL of 0.1 M cold phosphate buffer (pH 7.4) with 1 mM EDTA for 10 min, and the homogenate obtained was centrifuged at 3000 rpm for 15 min. The analysis was performed using the clear supernatant obtained.

For histopathological investigation, parts of the tissues were fixed in 10% formalin, embedded into paraffin, sectioned, and finally stained with hematoxylin and eosin.

Salivary NO estimation by Griess method (19):

The determination of the NO levels is based on the transformation of nitrate to nitrite with the aid of an enzyme. It is a diazotization reaction in which a nitrosating agent is produced by acetified NO. The nitrosating agent reacts with sulfanilic acid, resulting in the formation of a diazonium ion. This ion is coupled

with N-(1-naphthyl) ethylenediamine to produce the chromophoricazo derivatives, which absorb light at 540–570 nm. The reagents used included N-(1-naphthyl)-ethylene diamine (NED) dihydrochloride and sulphanilamide solutions. The sulphanilamide solution was prepared by mixing 0.5 g of sulphanilamide in 100 mL of 20% v/v hydrochloric acid. The N-(1-naphthyl)-ethylene diaminedihydrochloride solution was prepared by mixing 0.3 g of a solid reagent (NED dihydrochloride) in 100 mL of 1% v/v hydrochloric acid. A standard NO solution was pipetted out into 5 different test tubes in the range of 0.2–1 mL. Distilled water was used to increase the volume of each test tube by 1 mL. A separate test tube was taken with 1 mL distilled water, which served as blank. One milliliter each of the sulphanilamide solution and NED dihydrochloride solution were added to each of the test tube and the solution was mixed. The test tubes were later incubated at the room temperature for 10 min, and the absorbance was read at 550 nanometer. For test sample, 100 µL of the saliva/tissue sample was taken in a test tube. The volume was prepared up to 1 mL with 0.9 mL of distilled water. Then, 2 mL of sulphanilamide solution was included and kept for 5 min. After 5 min, 2 mL of NED dihydrochloride solution was added. After 10 min, the absorbance was measured at 550 nm. The concentration was then calculated from a calibration plot prepared from a series of standard nitrite. The calculation was performed as follows:

0.1 mL of sample contained=------- µM/L of NO.
∴ 1000 mL of sample contained=----- µM/L of NO.

Statistical Analysis

The data was analyzed by using the *SPSS* software version 17 (SPSS Inc.; Chicago, IL, USA). Data obtained were analyzed using independent Student's t-test for comparison between the groups, while Chi- Square Test was used for the association of the age and gender with different parameters. Pearson's correlation was used for analyzing correlations between the groups.

RESULTS

The mean age of Group I subjects was 29.1 years. It included 45% (9/20) and 55% (11/20) of female and male subjects, respectively. The mean age of Group II subjects was 55.45 years. It included 35.0% (7/20) and 65.0% (13/20) of female and male subjects, respectively (Table 1).

The mean salivary NO levels in Group I was 78.59 µM/L, while the mean salivary NO levels of Group II was 115.6765 µM/L. The mean tissue NO levels in Group I was 87.6315 µM/L, while it was 172.376 µM/L for Group II. Comparison of the salivary NO levels between Group I and Group II showed that that salivary NO levels were higher in Group II with a t value of –27.685, which was statistically significant with a p value of <0.001. Comparison of the tissue NO level between Group I and Group II showed that the tissue NO level was higher in Group II with a t value of –180.863, and it was statistically significant with a p value of <0.001 (Table 2).

Fair correlation was noted between the salivary and tissue NO levels in Group I (r=0.42), and excellent correlation was observed between the salivary and tissue NO levels with p<0.001, which is significant at an error of <1% (r=0.99). The overall correlation was found to be excellent between the salivary and tissue NO levels. (r=0. 99 in OSCC) (Table 3, Graph 1).

DISCUSSION

Highly RNS and reactive oxygen species (ROS) are involved in the process of cancer formation of the oral cavity (20). The presumption behind this hypothesis is that these free radicals could damage the cellular materials, resulting in the initiation or transformation of the normal cells into malignant ones (21). The extent

Table 1. Comparison of study participants according to gender

	Group			Chi-Square Test	
Gender	Normal	OSCC	Total	Chi-Square Value	p-value
Male	11	13	24	0.42	0.52(NS)
	55.0%	65.0%	60.0%		
Female	9	7	16		
	45.0%	35.0%	40.0%		
Total	20	20	40		
	100.0%	100.0%	100.0%		

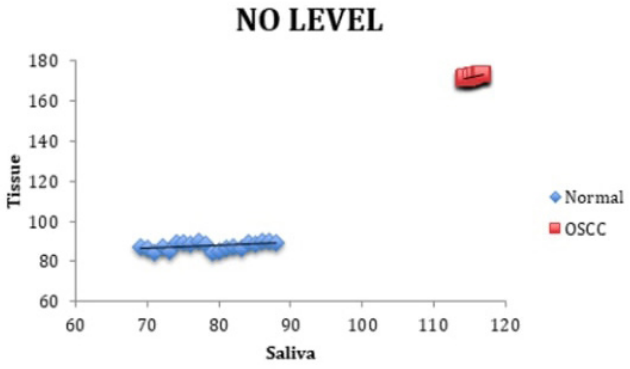
Table 2. Comparison of age and nitric oxide levels between the study groups

	Group	N	Mean	Std. Deviation	Mean Difference	95% Confidence Interval of the Difference		t	df	p-value
						Lower	Upper			
Age	Normal	20	29.10	5.83	–26.35	–31.44	–21.26	–10.48	31.29	<0.001*
	OSCC	20	55.45	9.62						
Saliva	Normal	20	78.59	5.92	–37.09	–39.80	–34.37	–27.69	19.97	<0.001*
	OSCC	20	115.68	0.94						
Tissue	Normal	20	87.63	1.92	–84.74	–85.69	–83.80	–180.86	26.16	<0.001*
	OSCC	20	172.38	0.85						

Table 3. Correlation between tissue and salivary NO levels

	r	p–value
Normal	0.42	0.07
OSCC	0.99	<0.001*

Graph 1. Correlation between tissue and salivary NO levels



of this destruction is also influenced by the defense mechanisms of the body against these free radicals interceded by various other cellular antioxidants. RNS could lead to the activation of pro carcinogens, inactivation of enzyme repair systems, initiation of lipid peroxidation process, and damage to the DNA, thereby affecting the triggering and promotion mechanism of carcinogenesis. The prime source of entire RNS is NO in the biological systems (22). The range of operations of NO are broad, elaborated, and multifarious in cancer biology.

Several studies with serum have been conducted to estimate the NO level in oral cancer patients. However, studies using saliva or tissue samples to estimate the NO levels in OSCC subjects are limited in the existing literature.

The comparison of the salivary NO levels between the groups in our study showed that salivary NO level was higher in the study group. This observation was in line with the research conducted by Bahar et al. (23), which demonstrated an alteration in the salivary composition of patients with OSCC when compared to the healthy subjects. The enhanced salivary NO levels could be attributed to the increased dietary NO from tobacco and its components (24). Dietary NO is absorbed from the upper gastrointestinal tract. It enters the saliva through the salivary glands through an active transport mechanism after getting actively concentrated from the plasma. Furthermore, in oral cancer patients, the NO level is increased due to the overexpression of enzyme inducible NO synthase (iNOS). Malignant epithelium of oral cancer or oral cancer induced inflammatory response are believed to be the possible sources of iNOS (24).

The tissue NO levels between the groups demonstrated that the tissue NO levels were higher in subjects with OSCC than in healthy individuals. This finding was in accordance with the study conducted by Korde et al. (25), where the tissue as well as the serum NO levels were significantly increased in subjects with OSCC. The increase in the NO levels may be described on the basis that there could be a generalized increase in the synthesis of NO throughout the body of the cancer patient or it could reflect elevated degradation of NO (25). This enhanced production of NO products is generally assisted by oxidative stress. The interaction of NO either with superoxide or oxygen leads to the formation of reactive nitrogen oxide species, which in turn can mediate either oxidative or nitrosative stress (25). NO forms peroxynitrite (ONOO) at a higher concentration, which is a potent oxidant that plays a major role in the initiation of oral cancer (25).

Furthermore, the present study was also in accordance with those conducted by Connelly et al. (26), where the authors reported significantly elevated levels of iNOS mRNA and NO production in OSCC cases when compared with oral dysplasias and normal cases. It is believed that the expression of iNOS and NO production play a role in the growth and invasion of oral carcinoma mostly through an angiogenic mechanism. The growth and invasion process of the carcinoma is determined by the proliferation of new vessels arising from the surrounding stroma. This growth of new vessels is mediated by iNOS through NO, which functions as a cellular signal for angiogenesis (26).

Gallo et al. (27) conducted a study that demonstrated significantly increased levels of total NOS in the tissues of the OSCC group when compared with the control group. Their study also demonstrated elevated levels of iNOS and cGMP in the tumor tissue specimens when compared with that in normal mucosa samples. In addition, enhanced total NOS activity was observed in cases where lymph node metastasis occurred. The authors concluded that the NO pathway appeared to stimulate tumor angiogenesis and help spread the tumor in patients with head and neck malignancies (27).

A study by Gokul et al. (28) also showed that the levels of NO and malondialdehyde were significantly increased in OSCC cases in both blood and tissue specimens, specifying elevated oxidative stress in OSCC patients with a restricted antioxidant defense mechanism. The raised oxidative stress in OSCC patients is indicated by the increased levels of MDA and NO. This disproportion in the status of the oxidant–antioxidant can be regarded as one of the elements liable for the pathogenesis of oral cancer.

In the present study, the correlation of salivary NO with tissue NO levels in the 2 groups was also analyzed, which makes this study unique in the current time. Our study highlights the increased expression of NO in the saliva and tissue specimens of subjects with OSCC when compared with those of healthy individuals. Moreover, an excellent correlation was noted between salivary and tissue NO levels in subjects with OSCC. These results suggest that NO could be used as a potential biomarker of OSCC.

Only a limited number of studies have been conducted until date for assessing the role of NO in the pathogenesis of OSCC. Most of the studies have reported the tumor-promoting effects of NO due the damage of the DNA, which is considered as one

of the causative factors for oral cancer development, which is in accordance with our present study as seen in cases of OSCC.

As significant increase was observed in the levels of NO in the saliva and tissue specimens, with excellent correlation in OSCC; this study suggests the use of salivary NO as a potential diagnostic marker in OSCC.

The basic elements for an effective diagnostic technique include the following: ease of use, minimal discomfort to patients, and collection of sufficient evidence for investigation. Basically, a diagnostic procedure should not be complex or time consuming. High sensitivity and potential for automation are other desirable requirements. In addition, avoidance of false-positives results and therefore reducing the anxiety of the patients and the need for additional investigations or unnecessary treatments are also required. Therefore, in the present study, we used saliva and tissue samples for the assessment of the NO levels, which optimally meets all of the above requirements.

Non-invasive methods such as the analysis of saliva and reliable methods such as the analysis of tissues will provide a cost effective approach for screening a large population in the near future.

Extensive studies with larger sample size are required to assess the actual role of NO in the initiation and promotion of OSCC along with testing of the utility of the NOS enzyme inhibitors as chemo-preventive agents in order to minimize the risk of human cancer. Elaborate studies with a larger sample size are also required to establish the effectiveness of using NO as an adjunctive diagnostic marker in OSCC.

CONCLUSION

The encouraging results of present study demonstrate the possible involvement of NO in the pathological process of OSCC as noticeable from elevated NO level in the saliva and tissues of OSCC patients. Thus, the findings of the present study indicate that NO can be used as an adjunctive diagnostic marker by evaluating the salivary and tissue NO levels in OSCC. Furthermore, it could provide a profitable approach for screening huge population groups in the future.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Nitte University (certificate number ABSM/EC/97/2014).

Informed Consent: Informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - R.G.; Design - R.G.; Supervision - S.G.B. Resources - R.L.C.; Materials -R.G.; Data Collection and/or Processing - R.G.; Analysis and/or Interpretation - R.G.; Literature Search - B.B.; Writing Manuscript - R.L.C.; Critical Review - B.B.

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