ORIGINAL RESEARCH

Estimation of Salivary Nitric Oxide Level in Various Stages of Precancerous Lesion

Ajit Mishra[1], Arti Mishra[2]

ABSTRACT

Introduction: Oral cancer accounts for 5.6% of all cancers in populations, and the most prevalent form of oral cancer is squamous cell carcinoma. Although many measures exist to treat it, the survival rate is still undesirable. Nitric oxide, a biological messenger, has been implicated in the progress of many diseases including cancer. Bodis and Haregowin reported that freshly released human saliva contains measurable and sometimes relatively high levels of nitric oxide.

Conclusion: In this study, the nitric oxide level has been estimated in saliva to open a new era for the early diagnosis and effective management of oral squamous cell carcinoma.

Keywords: Nitric oxide, Oncogenesis, Oral cancer.

How to cite this article: Mishra A, Mishra A. Estimation of Salivary Nitric Oxide Level in Various Stages of Precancerous Lesion. Int J Oral Care Res 2018;6(2):56-59.

Source of support: Nil

Conflicts of interest: None

INTRODUCTION

Carcinogenesis is a multistep process and is influenced by various mutagenic insults in growth and development of neoplasia. These mutational events may promote tumor growth through sequential activation or inactivation of various genes within a cell, leading to increased expression of oncogene proteins escaped either from cell cycle regulatory mechanism or from immune surveillance. However, the molecular mechanism of the mutational events leading to head-and-neck squamous cell carcinoma remains completely elucidated.[1]

Oral cancer accounts for 5.6% of all cancers in populations, and the most prevalent form of oral cancer is squamous cell carcinoma.[2] Oral squamous cell carcinoma is the sixth most common human cancer, with an increasing incidence in younger people with reported the high morbidity rate and a 5 years mortality rate of about 50%.[3] Although many measures exist to treat it, the survival rate is still undesirable.[2]

Since the discovery of nitric oxide as a biological messenger in 1987, it has been implicated in many disease processes ranging from septic shock to cancer.[4] In living tissue nitric oxide is synthesizing from the terminal guanidine group of the amino acid L- arginine by the enzyme nitric oxide synthase (NOS), and there are at least three NOS isoenzymes, each being the product of a distinct gene.[5]

NOS converts L- arginine to L- hydroxyarginine and subsequently to nitric oxide and citrulline. As electron oxidation of one N- atoms of the guanidine of L-arginine to nitric oxide and citrulline through the cofactors, NADPH, flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin. Moreover, the precise role of each cofactor remains unresolved.[6]

Bodis and Haregowin reported that freshly released human saliva contains measurable and sometimes relatively high levels of nitric oxide, whereas Ohashi *et al*. evaluated elevated production of salivary nitric oxide in oral mucosal diseases in their study. However, still, the cellular origin, the normal concentration, and the physiological and possible role of nitric oxide in saliva are at present unknown.[7]

Nitric oxide may have both beneficial and detrimental action in neoplastic tissues. It has been implicated as having a positive role in permitting tumor growth, including mutagenicity, angiogenesis, and metastasis, although it has been implicated in the cytotoxicity of macrophages toward tumor cells and in immunosuppression.[8] This apparent controversy is probably explained by the nitric oxide concentrations that are found in different experimental and clinical models. It is likely that nitric oxide levels produced in human cancers are insufficient to cause apoptosis and cell death, but instead, facilitate the process of angiogenesis and tumor dissemination.[9]

The present study has been undertaken to measure the level of salivary nitric oxide to establish its role as a potential marker in the transformation of pre-cancerous lesion into malignancies.

[1]Reader, Senior Lecturer[2]

[1]Department of Oral Medicine and Radiology, Maitri College of Dentistry and Research Centre, Anjora, Durg, Chhattisgarh, India

[2]Department of Orthodontics and Dentofacial Orthopedic, Maitri College of Dentistry and Research Centre, Anjora, Durg, Chhattisgarh, India

Corresponding Author: Dr Ajit Mishra, Dutta Colony, Near Rajmohni Bhawan, In front of PG College, Ambikapur, Chhattisgarh, India. Tel.: 07828185565/7869405405. e-mail: drajitmishra@gmail.com

Aims and Objectives

The aims and objectives of the present study are to estimate and correlate the salivary nitric oxide levels in oral pre-cancer and to evaluate the possibility of any role of salivary nitric oxide as a parameter during the transformation of pre-cancerous lesion and conditions into oral cancer.

MATERIALS AND METHODS

The study was conducted in the Department of Oral Medicine and Radiology of Modern Dental College & Research Center, Indore M.P. India. The study got the approval by the ethical committee after screening.

The participants included in the study signed on informed consent.

Group 2–50 individuals with clinically and histopathologically diagnosed cases of oral pre-cancer. These patients were also subdivided into three age groups.

- Group A - patients in age range of 20–35
- Group B - patients in age range of 36–50
- Group C - patients in age range of 51–65.

Furthermore, the case selections were done on the basis of following.

Inclusion criteria included patients in age range of 20–65 years, patients without having any other systemic disorder, patients in whom the malignancy originated in the oral mucosa, and patients not receiving any radiotherapy or chemotherapy for cancer.

Patients and control participants with diabetes mellitus, liver disease, and rheumatoid arthritis, and patients on any medical treatment for major illness including supplementation of antioxidants were excluded from the study and other exclusion criteria included patients who had undergone any type of major surgery in last 6 months, previously treated cases of oral cancer and patients with periapical lesions.

The clinical information was recorded in a definite format. The careful intraoral examination was carried out, and the findings were recorded in the Performa. Informed consent of the patient for the study was obtained. Unstimulated whole saliva samples were collected from patients for the study and biopsies were taken to establish the histopathological diagnosis.

Collection of Saliva Sample

The salivary samples were collected before the biopsy. The participants in the experimental groups were asked to rinse their mouth with betadine for 2 min which ensured a substantial reduction in bacterial count. The patients were then asked to wait for a minute, after which freshly secreted unstimulated saliva, about 2 mL was collected in a wide mouth sterile container by spitting method. The sample was then centrifuged for 5 min at 3000 rpm, and the supernatant fluid was then stored at −20°C until use.[3,7]

Estimation of Salivary Nitric Oxide

Principle

This method is based on the estimation of nitrite levels in saliva. Whole saliva is mixed with the "Griess reagent" (1% sulfanilamide 0.1% naphyl ethylene diamine dihydrochoride, 2.5% H_3PO_4) which gives a purple color to the solution which was then read on a photoelectric colorimeter in a green filter. The intensity of the purple coloration is directly proportional to the amount of nitrite level present in the saliva.

Reagents

Sulphanilamide, N-naphthylene ethylene diamine, standard sodium nitrite solution, ortho-phosphoric acid, distilled water.

Equipment required for the assay as follows: Test tubes with test tube stand, centrifuge machine, micropipette, photoelectric colorimeter, and cuvette.

Establishing the Standard

A standard curve has to be obtained using a known standard solution of the substance to be determined reacted with an appropriate reagent so that unknown concentration of the substance to be determined can be obtained from the standard curve by a colorimeter. The substance to be determined in our study was nitrite, and hence, known concentration of sodium nitrite solution was prepared for evaluation.[7]

Procedure

Three test tubes were taken and labeled as "blank" (B), "standard" (S), and "test" (T). 1.0 mL of distilled water was taken in a test tube labeled as "B." 1.0 mL of standard working solution of nitrite was taken in a test tube labeled as "S." 1.0 mL of whole saliva sample was taken in a test tube labeled as "T." Sulphanilamide and naphthylene ethylene diamine hydrochloride were mixed in a separate test tube to form a "Griess reagent," and this was left for 10 min so that complete reaction can take place. Within the available time, the colorimeter was calibrated to 00 after placing the distilled water filled cuvette in it. Then 0.1 mL of "Griess reagent" was mixed with the "blank" and it was left for 10 min till the reaction took place. Then, the reading was taken on the colorimeter in the green filter. Next 0.1 mL of Griess reagent was mixed with the standard, and it was left for 10 min till the reaction took place. Then the reading was

taken on the colorimeter in the green filter. In next step, the Griess reagent was mixed with the test (0.1 mL of whole saliva), and it was also kept for 10 min to complete the reaction. Moreover, after 10 min reading was taken on Colorimeter.

RESULTS

The present study was conducted to study the role of nitric oxide in carcinogenesis. A total of 50 patients were studied of which 50 were oral pre-cancer patients. The clinical status of patients was confirmed by histopathological examination. Patients of this groups were divided into three age groups 20–35, 36–50, and 51–65, named A, B, and C, respectively.

Figure 1 depicts the descriptive statistics of parameter "nitric oxide level estimation in pre-cancerous subjects in different age groups. The mean salivary nitric oxide level in 20 pre-cancerous patients within age Group A was found to be 118.0 ± 51.75 µg/mL with a range of 69.9–274.3 µg/mL. The mean nitric oxide level of 17 pre-cancerous patients in the Group B was found to be 164.2 ± 78.90 µg/mL with a range of 86.0–355.0 µg/mL. In age Group C, the mean total nitric oxide was found to be 166.7 ± 83.16 µg/mL with a range of 80.7–328.1 µg/mL.

DISCUSSION

Cancer of the oral cavity presents challenging and unresolved problems for the human population. One of the most intensive investigative efforts of the cell and molecular biology today are devoted to the elucidation of the mechanisms that lead to genetic mutations and faulty regulation of cell proliferative processes resulting in oral cancer.[10,11] The crucial and ultimate step leading to carcinogenesis is DNA damage and angiogenesis.[10,11]

Nitric oxide level also can be evaluated in serum and tissue but saliva was considered as a better option than serum because of collection of saliva is a noninvasive and psychologically good for the patients furthermore presence of nitric oxide has been proved by many studies,[12-14] and its role in cancer is also studied;[15] however, nitric oxide in saliva has never been estimated in oral cancer.

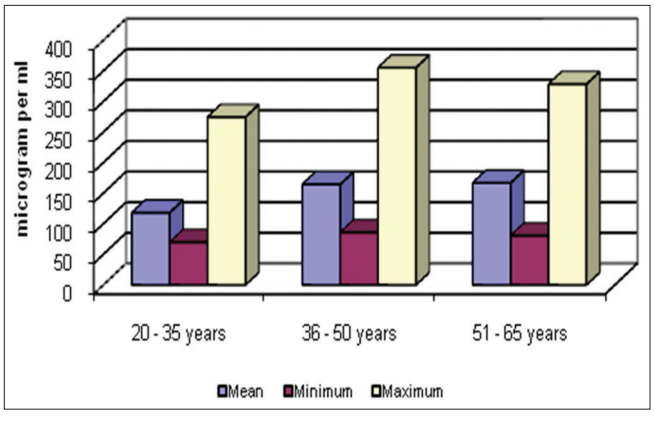

Figure 1: Nitric oxide levels in pre-cancerous subjects of different age groups

Biopsy samples of human breast cancer showed the presence of greater expression of iNOS.[16] Brennan *et al*., reported the significantly higher expression of iNOS in oral squamous cell carcinoma tissue, linking it with high levels of nitric oxide in its pathogenesis.[17]

Accordingly, we measured levels of salivary nitric oxide of oral cancer patients. As nitric oxide is highly unstable and has a very short half-life, levels of nitrite were used to estimate the levels of total nitric oxide formation.

The mean nitric oxide level in healthy control (histopathological no dysplasia) in our study was 132.5 µg/mL with the range of 69.9–258.2 µg/mL as comparable and similar to other studies performed by Supriya 2004 and Venerssa 2007.

The mean nitric oxide level in patients with pre-cancer (histopathologically with dysplasia) in this study was 146.37 µg/mL with the range of 69.9–328.1 µg/mL. The level of nitric oxide in pre-cancerous group was found significantly higher than the normal healthy group, which supports the hypothesis of nitric oxide activity in affected subjects.

A negative feedback loop seems to exist between nitric oxide production and p53 tumor suppressor gene.[8] P53 is a tumor suppressor gene which plays a crucial role in cell immortalization or cell death. Participate in the cell cycle regulation at g1/s and g2/m checkpoint. Increased p53 triggers the activation of downstream p21 latter arrested cell cycle.[18]

Dysplastic lesions display abundant capillaries leading to intense vascularization in invasive squamous cell carcinoma and a tumor with increase in size requires angiogenesis. Nitric oxide plays an important role in angiogenesis.[9,10]

The specific action of nitric oxide is known to be concentration dependent, and the levels found in human are thought to be responsible for enhanced angiogenesis and tumor aggressiveness.[9,10] This may be the reason why the nitric oxide levels are increasing in cancerous patients because the tumor is consistently growing and it needs angiogenesis which requires nitric oxide.

The another mechanism that can explain the increase in nitric oxide level in cancerous patients is because of mutation of p53 gene which was seen in 54–67% of carcinoma patients.[18] And mutated p53 known to cause an increase in nitric oxide level.[8,19]

In pre-cancerous group mean salivary nitric oxide level was 118.0 µg/mL, 164.2 µg/mL and 166.7 µg/mL

in Group A (20–35 years), B (36–50 years) and C (51–65 years), respectively.

It was observed that the mean nitric oxide level was increasing with age in cancerous patients. The estimated mean value for Group A was 340.9 µg/mL, for B group 368.4 µg/mL and it was 404.9 µg/mL for Group C.

Thus a gradual increase in the levels of nitric oxide exists in relation to initiation and promotion of carcinogenesis, and through this study, an effort was made to explore the mechanisms operating in oral carcinogenesis. Hence, nitric oxide has already made it from the bench to the bedside, and it is not unlikely that new development in this area will drastically change cancer treatment during the coming years.

Although we made our sincere efforts to establish the role of salivary nitric oxide in pre-cancer Still, the further studies on this should remain continued in the field of oral medicine to establish salivary nitric oxide as a diagnostic and prognostic marker to open a new era in the management of oral malignancies.

Summary

It is commonly held that a myriad of cellular interaction influences the development and growth of neoplastic diseases. However, within the past decade, significant effort has focused on various mutagenic insults hypothesized to contribute to the multi-step process of carcinogenesis.[1]

Based on the results of the study, the following conclusions were drawn:

A. There could be potential role of nitric oxide in the pathogenesis of oral pre-cancer and oral cancer.
B. Increased salivary nitric oxide in pre-cancer patients was not because of age, but it was found to be associated with progression of diseases.

REFERENCES

1. Bentz BG, Haines GK 3rd, Lingen MW, Pelzer HJ, Hanson DG, Radosevich JA, *et al.* Nitric oxide synthase Type 3 is increased in squamous hyperplasia, dysplasia, and squamous cell carcinoma of the head and neck. Ann Otol Rhinol Laryngol 1999;108:781-7.
2. Zhao S, Tong X, Zhu F. Nitric oxide induces oral squamous cell carcinoma cells apoptosis with p53 accumulation. Oral Oncol 2005;41:785-90.
3. Bahar G, Feinmesser R, Shpitzer T, Popovtzer A, Nagler RM. salivary analysis in oral cancer patients DNA and protein oxidation, reactive nitrogen species, and antioxidant profile. Cancer 2007;109:54-9.
4. Brennan PA, Thomas GJ, Langdon JD. The role of nitric oxide in oral diseases. Arch Oral Biol 2003;48:93-100.
5. Shang ZJ, Li JR, Li ZB. Effect of exogenous nitric oxide on oral squamous cell carcinoma: An *in vitro* study. J Oral Maxillofac Surg 2002;60:905-10.
6. Bian K, Murad F. Nitric oxide-bio-generation, regulation and relevance to human diseases. Front Biosci 2003;8:d264-78.
7. Sunita M, Shanmungam S. Evaluation of saliva nitric oxide levels in oral mucosal diseases: A controlled clinical trial. Indian J Dent Res 2006;17:117-20.
8. Hsuen CY, Lin LM. Expression of inducible nitric oxide synhsase in human oral premalignant epithelial lesion. Arch Oral Biol 2002;47:387-92.
9. Brennan PA, Umar T, Wilson AW, Mellor TK. Expression of type 2 nitric oxide synthase and vascular endothelial growth factor in oral dysplasia. J Oral Maxillofac Surg 2002;60:1455-60.
10. Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB, *et al.* The multifaceted roles of nitric oxide in cancer. Carcinogenesis 1998;19:711-21.
11. Brennan PA, Moncada S. From pollutant gas to biological messenger: The diverse actions of nitric oxide in cancer. Ann R Coll Surg Engl 2002;84:75-8.
12. Tenovuo T. The biochemistry of nitrates, nitrite, nitrosamines and other potential carcinogens in human saliva. J Oral Pathol 1986;15:303-7.
13. Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, *et al*. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat Med 1995;1:546-51.
14. Bodies S, Haregowin A. Evidence for the release and possible neural regulation of nitric oxide in human saliva. Biochem Biophys Res Commun 1993;194:347-50.
15. Brennan PA, Palacios-Callender M, Zaki GA, Spedding AV, Langdon JD. Type II nitric oxide synthase (NOS2) expression correlates with lymph node status in oral squamous cell carcinoma. J Oral Pathol Med 2001;30:129-34.
16. Bing RJ, Miyataka M, Rich KA, Hanson N, Wang X, Slosser HD, *et al.* Nitric oxide, prostanoids, cyclooxygenase, and angiogenesis in colon and breast cancer. Clin Cancer Res 2001;7:3385-92.
17. Brennan PA, Palacios-Callender M, Umar T, Tant S, Langdon JD. Expression of Type 2 nitric oxide synthase and p21 in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 2002;31:200-5.
18. Rajendran R, Shirley V. Inducible nitric oxide synthase expression is upregulated in oral submucous fibrosis. Indian J Dent Res 2007;18:94-100.
19. Zhao SF, Tong XY, Zhu FD. Nitric oxide induces oral squamous cell carcinoma cells apoptosis with p53 accumulation. Oral Oncol 2005;41:785-90.