DOI: 10.7860/JCDR/2016/16517.7126

Original Article

Dentistry Section

Evaluation of Salivary Nitric Oxide Levels in Smokers, Tobacco Chewers and Patients with Oral Lichenoid Reactions

S. PREETHI[1], JOY IDICULLA JOSE[2], B. SIVAPATHASUNDHARAM[3], B. SABARINATH[4]

ABSTRACT

Introduction: Nitric oxide (NO), a free radical, acts as a signalling molecule affecting numerous physiological and pathological processes. Role of nitric oxide as a mediator in tobacco related habits and the resultant oral lichenoid reactions was assessed.

Aim: The aim of the study is to evaluate and compare the salivary nitric oxide levels in normal patients with that of smokers, tobacco chewers and patients with oral lichenoid reactions.

Materials and Methods: One hundred and twenty patients were enrolled in the study which included 30 healthy patients without any chronic inflammatory lesion and habit as controls (group I), 30 smokers without the habit of tobacco/betel nut chewing and any oral lesion (group II), 30 tobacco chewers without the habit of smoking and any oral lesion (group III) and 30 histologically confirmed cases of oral lichenoid reaction with the habit of tobacco usage (group IV). Saliva from these patients was collected and the nitrite concentration was assessed.

Results: Our results concluded that there was highly significant increase in the nitric oxide levels in smokers, tobacco chewers and patients with oral lichenoid reactions compared to that of controls. Also, there was a significant increase in nitric oxide levels in patients with smoking associated oral lichenoid reactions in comparison with smokers and in patients with lichenoid reactions associated with tobacco chewing in comparison with tobacco chewers.

Conclusion: Estimation of salivary nitric oxide levels is a simple, non-invasive procedure and could be analysed to suggest the role of nitric oxide in the pathogenesis of these lesions. The increased activity of the enzyme may indicate that nitric oxide has a pathophysiological role in these lesions.

Keywords: Betel nut, Lichen planus, Saliva, Smoking

INTRODUCTION

Nitric oxide (NO), a free radical, is synthesised from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). There are three distinct isoforms of NOS, namely, nNOS (Type I, NOS-I and NOS-1) present in the neuronal tissue, iNOS (Type II, NOS-II and NOS-2) which is inducible in a wide range of cells and tissues and eNOS (Type III, NOS III and NOS-3), found in the vascular endothelial cells [1]. Nitric oxide, a signalling molecule, acts as a 'double-edged sword' affecting numerous physiological and pathological processes [2]. Nitric oxide is sourced into the body from both metabolic and dietary substances. It is then transported to the salivary glands via blood. Facultative anaerobic bacteria reduce the nitrate form to nitrite, which is a potential substrate for nitric oxide [3].

Low nitric oxide levels are associated with homeostatic actions such as immune functions, blood flow, platelet aggregation, neurotransmission, and memory, whereas excess nitric oxide production is involved in inflammatory and immunological disorders, pain, neurological diseases, atherosclerosis, and cancer [4]. It also plays an important role in the occurrence and progression of tumours, involving mechanisms such as DNA damage, inducing tumour angiogenesis and promoting tumour invasion and metastasis [5].

Numerous studies have been published on increased salivary nitric oxide levels and its possible role in the aetiopathogenesis of Oral lichen planus. However, in a country like India, our concern is more towards the usage of tobacco. Smoked and smokeless forms are very prevalent and sometime leads to Oral Lichenoid reactions. Tobacco habits increase the generation of free radicals and reactive oxygen species (ROS) and some constituents of tobacco can cause inflammation, DNA damage and cell death. In turn, these inflammatory cells act as a source of oxygen radicals. Various immunological mediators along with macrophages, T lymphocytes and natural killer cells stimulate iNOS which is capable of producing nitric oxide for a long period of time [6]. Hence, the study was undertaken to evaluate the possible role of nitric oxide in the aetiopathogenesis of the resulting oral lichenoid reaction.

MATERIALS AND METHODS

This study was conducted at the outpatient Departments of Oral Pathology and Microbiology and Oral Medicine and Radiology, Meenakshi Ammal Dental College and Hospital, Chennai, Tamil Nadu, India. The subjects comprised of four groups; Group I comprised of 30 subjects as healthy controls, Group II comprised of 30 subjects with smoking habit, Group III comprised of 30 subjects with tobacco chewing habit, Group IV comprised of 30 subjects with oral lichenoid reactions. The patients were in the age group of 20-50 years, without any medically compromised conditions or systemic illness. Group I had 12 males and 18 females, group II had 30 males with only smoking habit, group III with 26 males and 4 females with only tobacco chewing habit and group IV had 22 males and 8 females. Ten patients in group IV had the habit of smoking (group IVa), 16 patients with the habit of chewing tobacco (group IVb), and the rest 4 with both smoking and tobacco chewing habits (group IVc).

Written consent of the patient was obtained and a biopsy was performed to confirm the clinical diagnosis of lichenoid reaction with histopathological findings. The subjects in the experimental groups were asked to rinse their mouth with povidone iodine mouth rinse for 2 minutes. After waiting for a minute, freshly secreted unstimulated saliva, about 1ml was collected in a sterile container. The sample was then diluted with 10ml of phosphate buffered saline to neutralise the pH. The mixture was then centrifuged for 5 minutes at 3000 rpm and the supernatant fluid was stored at 20°C until use. The substrate to be determined in our study was nitrite and hence, known concentrations of sodium nitrite solutions were prepared for evaluation. Sodium nitrite solutions in varying concentrations from 10µg-50µg were prepared and test tubes were labelled as S1 – S5 respectively. Aliquots of these solutions (whose

concentration were known) were reacted with Griess reagent which was prepared by using 1% Sulfanilamide, 0.1% Napthylethylene Diamine Dihydrochloride, 2.5% Phosphoric acid and incubated at room temperature for 10 mins to ensure complete reaction takes place. The purplish pink colour thus obtained was read in spectrophotometer and the absorbance optical density (O.D) was recorded. This was taken as standard optical density (SOD).

In the same way, 1ml of the saliva sample were taken from each patient in our study group and was incubated with an equal volume of Griess reagent in a test tube for 10 minutes at room temperature. The colour change in the test tube was read in a spectrophotometer at 545nm and absorbance was recorded. This represented the test optical density (TOD). The concentration of nitrite in the saliva was calculated using the formula

TOD/SOD x concentration of the standard solution.

The levels of nitric oxide were recorded for controls and the study groups.

Ethical Approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The written consent form was approved by the ethics committee.

RESULTS

Multiple comparisons between groups using One-Way ANOVA and using Post-HOC Tukey HSD analysis were done using version 16.0 of SPSS software. Values were found to be statistically significant (p-value <0.05) for all the parameters taken into consideration. In group I, the salivary nitric oxide levels were in the range from 3.04µg/dl to 5.186µg/dl with a mean of 3.928µg/dl and in group II, the salivary nitric oxide levels were in the range from 6.08µg/dl to 7.154µg/dl with a mean of 6.648µg/dl. Group III had salivary nitric oxide levels in the range from 5.768µg/dl to 6.841µg/dl with a mean of 5.929µg/dl and patients in group IV had the salivary nitric oxide levels were in the range from 8.808µg/dl to 16.006µg/dl with a mean of 11.880µg/dl [Table/Fig-1-3].

There was a significant increase in the nitric oxide levels among the four groups with maximum values seen in group IV consisting of patients with oral lichenoid reactions. However, the increase was not significant among group II and group III. Comparisons between group II (smokers) and group IVa (smoking associated with lichenoid reactions) showed a definite increase in values. Moreoever, Group III (tobacco chewers) were compared with group IVb (tobacco chewing associated with lichenoid reactions) and nitric oxide levels were increased in Group IVb. Intra comparisons between Group IVa and IVb were done and the value was increased in the latter and was stastically significant.

Group	N	Mean	Std. Dev	p-Value
Group 1	30	3.9284	0.5255	<0.001
Group II	30	6.6475	0.3343	
Group III	30	5.9294	1.0505	
Group IV	30	11.8796	1.8001	
Total	120	7.0962	3.1377	

[Table/Fig-1]: Comparison of Concentrations of Sodium Nitrite in saliva (µg/dl) using One-way ANOVA.
p ≤ 0.05 = significant.

(I) Group	(J) Group	Tukey HSD Q statistic	p-Value
Group I	Group II	13.6927	<0.001
	Group III	10.0764	<0.001
Group II	Group IV	40.0399	<0.001
	Group III	3.6163	0.155
Group III	Group IV	26.3472	<0.001
	Group IV	29.9635	<0.001

[Table/Fig-2]: Comparison of Concentrations of Sodium Nitrite in saliva (µg/dl) using Post-HOC Tukey HSD analysis.
p ≤ 0.05 = significant

(I) group	(J) group	Tukey HSD Q statistic	p-value
Group IVa	Group II*	14.0953	<0.001
	Group IVb	4.8020	<0.001
group IVb	Group III	25.8295	<0.001

[Table/Fig-3]: Comparison of Salivary sodium nitrite concentrations in Smokers, Tobacco chewers, patients with oral lichenoid reactions with smoking and patients with oral lichenoid reactions with tobacco chewing using Post-HOC Tukey HSD analysis.
p ≤ 0.05 = significant.

DISCUSSION

Lichenoid reaction, similar to oral lichen planus is an inflammatory lesion with inflammatory cells such as lymphocytes and plasma cells distributed in the stromal tissue. Since, the usage of tobacco products and oral lichenoid reactions produce an inflammatory reaction, cells like T lymphocytes and macrophages serve as a source of nitric oxide, thereby causing cell injury and damage. Nitric oxide can react with other radicals to form cytotoxic compounds, such as peroxynitrite, which can cause DNA damage and protein modifications by formation of carcinogenic nitrosamines or by inhibiting DNA repair mechanism [7,8]. It can also react directly with a variety of enzymes and other proteins to either activate or inhibit their functions. Inducible nitric oxide synthase (iNOS) is capable of producing sustained concentrations of nitric oxide in the micromolar range and hence, this molecule is principally involved in inflammatory processes and cancer formation [9].

In the present study, the levels of nitric oxide in the saliva of patients with smoking, tobacco nut chewing habits and patients with oral lichenoid reactions was compared with healthy controls. Nitric oxide levels can be estimated from blood, exhaled air, saliva, urine and also from polymerase chain reaction (PCR) and immunohistochemical analysis. In this study, saliva was used for the estimation of nitric oxide level as it was readily available, easier to collect and the procedure was non-invasive. The nitric oxide levels in saliva were measured by a calorimetric assay based on Griess reagent, described by Ohashi et al., [10].

The results obtained in the present study showed elevated salivary nitric oxide levels in smokers, tobacco chewers and patients with oral lichenoid reactions when compared to normal controls. An increase in nitric oxide levels were also seen in patients with lichenoid reactions when compared with smokers and tobacco chewers. The results even showed elevated levels of nitric oxide in patients with tobacco chewing associated oral lichenoid reactions with oral lichenoid reaction patients associated with smoking and this was also found to be statistically significant (p ≤ 0.05).

The results imply that tobacco smoking, tobacco chewing habit and lichenoid reactions can induce inflammatory changes in the oral mucosa. Chronic inflammation would play an important role in causing genetic damage and inducing tissue proliferation by oxidative damage of the DNA products derived from inflammation which induces enzymes, such as nitric oxide synthase, which in turn produces nitric oxide [11].

The values of salivary nitric oxide levels among the healthy controls correlated with the values of the control group in the study conducted by Sunitha M and Shanmugam [6]. Bodis and Haregewoin, found reduced salivary nitric oxide levels in smokers compared to control subjects [12]. This was in contrast with the present study which showed an elevated nitric oxide levels in smokers compared to controls. This could probably be due to different products of nitric oxide that were analysed. Nitrogen dioxide (NO_2) was evaluated in the former study compared to nitrite evaluated in our present study.

Fresh cigarette smoke contains nitric oxide primarily with little or no nitrogen dioxide [13]. Also, hypoxia is a synergistic inducer of iNOS expression and hence, favours the reaction between nitric oxide and oxygen resulting in nitrosative modifications [14]. This could explain the reason for increased nitric oxide levels in smokers in the present study compared to the former.

Ohashi et al., have reported that increase in salivary nitric oxide levels in oral lichen planus and recurrent apthous ulcerations is a consequence of cell damage [10]. Sunitha M and Shanmugam S also found increased levels of nitric oxide in lichen planus compared to controls and concluded that free radicals represent one route of pathogenesis and excess of salivary nitric oxide may have a pathophysiological implication for erosive and ulcerative lesions in oral lichen planus and recurrent aphthous ulceration [6]. The present study show elevated nitric oxide levels in patients with tobacco usage and a more elevated status in patients with oral lichenoid reactions.

Inducible nitric oxide synthase expression was upregulated in oral submucous fibrosis in a study conducted by Rajendran R and Shirley Varkey and their study group consisted of patients with the habit of areca nut chewing [15]. Nair UJ et al., first demonstrated that aqueous extracts of areca-nut and catechu were capable of generating free radicals at specific pH that are produced during auto oxidation of polyphenols in saliva of the tobacco users [16]. In accordance, there was an increased nitric oxide levels in patients in group III and increased more in patients with oral lichenoid reactions associated with tobacco chewing habit as seen in group IV.

Lichenoid reactions are a form of type IV contact hypersensitivity reaction [17] and according to Ross et al., nitric oxide production has been shown to be increased by tenfold in hypersensitivity reaction [18] and the present study showed increased nitric oxide levels in group 4.

Continuous local irritation and trauma caused by betel quid and pan masala can generate chronic inflammation, oxidative stress and cytokine production. Invitro studies have demonstrated the adhesive nature of areca-nut particles to cultured oral mucosal cells, leading to morphological changes and membrane damage. Oxidative stress and the generation of reactive oxygen species can drive affected cells to proliferation, senescence or cell death. Components of areca nut stimulate the release of inflammatory mediators such as prostaglandin E2 (PGE2), interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α) from primary cultured human oral keratinocytes. Activation of complement was demonstrated invitro using aqueous extracts of loose leaf chewing tobacco, dry snuff and moist snuff [19]. Earlier studies have shown that pan masala and gutkha have clastogenic and carcinogenic effects [20].

In any lichenoid lesion, whether reactive or dysplastic, there is the presence of localized mononuclear cell infiltration within the superficial lamina propria [21]. These T lymphocytes in the lamina propria produces increased levels of interleukin-6 (IL-6) and granulocyte macrophage colony stimulating factors (GM-CSF) and can be stimulated to produce more tumour necrosis factor-α (TNF-α) by IL-1β, IL-6 and GM-CSF. These cytokines, interleukins and TNF are capable of activating T cells, which in turn produces more iNOS and thus contribute to the release of nitric oxide. It is seen that lichenoid reactions being inflammatory, show increased nitric oxide levels due to the release of various inflammatory mediators, and these levels can be further enhanced by tobacco and its products, which explains the reason why there is an increased nitric oxide levels in patients with oral lichenoid reactions associated with the tobacco chewing habit. Increased nitric oxide levels can in turn cause DNA modifications and damage, progressing the cell to malignancy.

Studies have been done on the level of nitric oxide in smokers, smokeless tobacco users, lichen planus and ulcerative lesions and mostly on tumours, but very few studies have been conducted on oral lichenoid lesions and its aetiopathogenesis [22-24]. This study was an attempt to analyse the role of nitric oxide in oral lichenoid reactions. The results in our study were analysed using a spectrophotometer which requires a minimum saliva sample of 1ml for estimation. If the quantity is less than 1ml, it has to be diluted so as to make the volume up to 1ml to be analysed in the spectrophotometer. By diluting this sample, chances of error may occur which can interfere with the accuracy of the result. This was overcome in the present study by collecting adequate saliva sample from patients.

CONCLUSION

Although, at present we cannot completely confirm whether the over expression of iNOS in smokers, tobacco chewers and lichenoid reactions is mediated via hypoxia or due to the release of cytokines by inflammatory mediators as a result of tobacco or betel nut. Usage of tobacco products has produced lichenoid reactions in the buccal mucosa and the reasoning, at the molecular level is still debatable.

Either increased levels of nitric oxide contributed to the development of these oral lichenoid lesions or whether these oral lesions contributed to elevated nitric oxide levels or if they have a synergistic effect on each other is still unknown. Although, it appears reasonable to suspect that the increased activity of the enzyme may, at least in part, indicate that nitric oxide has a pathophysiological role in these conditions.

ACKNOWLEDGEMENTS

I wish to thank Mrs. N S. Jagadeeswari, Department of Biochemistry, Meenakshi Ammal Dental College and Hospital, Chennai for providing technical guidance and helping me with laboratory procedures.

REFERENCES

[1] Aldertin WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J*. 2001;357(3):593-615.
[2] Crowell JA, Steele VE, Sigman CC, Fay JR. Is Inducible Nitric Oxide Synthase a Target for Chemoprevention? *Molecular Cancer Therapeutics*. 2003;2:815-23.
[3] Zetterquist W, Pedroletti C, Lundberg JO, Alving K. Salivary contribution to exhaled nitric oxide. *Eur Respir J*. 1999;13(2):327-33.
[4] Cirino G, Santagada V, Caliendo G. The nitric oxide related therapeutic phenomenon: a challenging task. *Curr Pharm. Des*. 2002;8(3):215 –31.
[5] Chen WL, Feng HJ, Li JS, Li HG. Expression and pathological relevance of inducible nitric oxide synthase in osteosarcoma of jaws. *Int J Oral Maxillofac Surg*. 2007;36(6):541-44.
[6] Sunitha M, Shanmugam S. Evaluation of salivary nitric oxide levels in oral mucosal diseases: A controlled clinical trial. *Ind J Dent Res*. 2006;17(3):117-20.
[7] Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide invitro. *Proc Nalt Acad Sci*. 1992;89(7):3030-34.
[8] Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB. The multifaceted roles of nitric oxide in cancer. *Carcinogenesis*. 1998;19(5):711–21.
[9] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA*. 1990;87(4): 1620-24.
[10] Ohashi M, Iwase M, Nagumo M. Elevated production of salivary nitric oxide in oral mucosal diseases. *J Oral Pathol Med*. 1999;28(8):355-59.
[11] Cortés-Ramìrez DA, Gainza-Cirauqui ML, Echebarria-Goikouria MA, Aguirre-Urizar JM. Oral lichenoid disease as a premalignant condition: The controversies and the unknown. *Med Oral Patol Oral Cir Bucal*. 2009;14(3):118-22.
[12] Bodis S, Haregewoin A. Evidence for the release and possible neural regulation of nitric oxide in human saliva. *Biochem Biophys Res Commun*. 1993;194(1):347-50.
[13] Church DF, Pryor WA. Free-Radical Chemistry of Cigarette Smoke and Its Toxicological Implications. *Environmental Health Perspectives*. 1985;64:111-26.
[14] Melillo G, Musso T, Sica A, Taylor LS, Cox GW, Varesio L. A Hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *The Journal of Experimental Medicine*. 1995;182(6):1683-93.
[15] Rajendran R, Varkey S. Inducible nitric oxide synthase expression is upregulated in oral submucous fibrosis. *Ind J Dent Res*. 2007;18(3):94-100.
[16] Nair UJ, Floyd RA, Nair J, Bussachini V, Friesen M, Bartsch H. Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA invitro with betel quid ingredients. *Chem Biol Interact*. 1987;63(2):157-69.

[17] Trivedy CR, Craig G, Warnakulasuriya S. The oral health consequences of chewing areca nut. *Addiction Biology*. 2002;7(1):115-25.
[18] Ross R, Gillitzer C, Kleinz R, et al. Involvement of NO in contact hypersentivity. *Int immunol*. 1997;10(8):61-9.
[19] Chang T, Chowdhry S, Budhu P, Kew RR. Smokeless tobacco extracts activate complement invitro: a potential pathogenic mechanism for initiating inflammation of the oral mucosa. *Clin Immunol Immunopathol*. 1998;7(3):223-29.
[20] Nair U, Bartsch H, Nair J. Alert for a epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: A review of agents and causative mechanisms. *Mutagenesis*. 2004;19(4):251-62.
[21] Rajendran R, Sivapathasundharam B (2009) 6th ed. *Shafer's textbook of Oral Pathology*. India: Elsevier publication.
[22] Patel JB, Shah FD, Shukla SN, Shah PM, Patel PS. Role of nitric oxide and antioxidant enzymes in the pathogenesis of oral cancer. *J Can Res Ther*. 2009;5(4):247-53.
[23] Kapoor C, Wadhwan V, Vaidya S, Malik S. Triology of nitric oxide, mast cell and stress in pathogenesis of oral lichen planus. *J Oral Maxillofac Pathol*. 2013;17(2):156–62.
[24] Mehdipour M, Taghavi Zenouz A, Bahramian A, Gholizadeh N, Boorghani M. Evaluation of serum nitric oxide level in patients with oral lichen planus. *J Dent Shiraz Univ Med Sci*. 2014;15(2):48-51.

PARTICULARS OF CONTRIBUTORS:
1. Senior Lecturer, Department of Oral Pathology, Meenakshi Ammal Dental College, Chennai, India.
2. Head of Department, Department of Oral Pathology, Faculty of Dentistry, Melaka Manipal Medical College, India.
3. Head of the Department, Department of Oral Pathology, Meenakshi Ammal Dental College, Chennai, India.
4. Reader, Department of Oral Pathology, Meenakshi Ammal Dental College, Chennai, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. S. Preethi,
Senior Lecturer, Department of Oral Pathology, Meenakshi Ammal Dental College, Chennai, India.
E-mail : drpreethiopath@gmail.com

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Aug 28, 2015**
Date of Peer Review: **Oct 12, 2015**
Date of Acceptance: **Dec 07, 2015**
Date of Publishing: **Jan 01, 2016**