Estimation and Comparison of Levels of Salivary Nitric Oxide in Patients with Oral Lichen Planus and Controls

Sapna Panjwani, Anjana Bagewadi[1], Vaishali Keluskar[1], Rohit Malik, Shalu Rai, Deepankar Misra

Department of Oral Medicine and Radiology, Institute of Dental Studies and Technologies, Kadrabad, District Ghaziabad, [1]Department of Oral Medicine and Radiology, Kles V.K Institute of Dental Sciences, Nehru Nagar, Belgaum, (5900010) Karnataka, India

Correspondence to:
Dr. Sapna Panjwani,
Senior Lecturer, Department of Oral Medicine and Radiology, Institute of Dental Studies and Technologies, Kadrabad, District Ghaziabad, India.
E-mail: dr.sapna28@gmail.com

Date of Submission: Jan 02, 2012

Date of Acceptance: Mar 27, 2012

How to cite this article: Panjwani S, Bagewadi A, Keluskar V, Malik R, Rai S, Misra D. Estimation and comparison of levels of salivary Nitric Oxide in patients with oral lichen planus and controls. Int J Prev Med 2013;4:710-4.

Original Article

ABSTRACT

Background: Normal salivary function is considered to be critical for the maintenance of healthy oral mucosa. Oral fluids provide an easily available, non-invasive medium for the diagnosis of wide range of diseases and clinical situations. The objective of this study was to estimate and compare salivary nitric oxide levels in patients with oral lichen planus and healthy individuals.

Methods: Saliva was collected by spitting method. Unstimulated whole saliva thus collected was refrigerated at 4°C, and processed within 24 h for the estimation of nitric oxide levels which was done using Griess reaction. The results were analyzed using Student's "*t*" test.

Results: There was statistically significant difference in the levels of salivary nitric oxide between the study and control groups.

Conclusions: The present study clearly indicates a significant increase in salivary nitric oxide levels in oral lichen planus patients as compared to controls.

Keywords: Nitric oxide, oral lichen planus, saliva

INTRODUCTION

Saliva is one of the most important body fluids and plays a critical role in preservation and maintenance of oral health. It not only aids in speech, mastication, and swallowing, but also has become a useful systemic sampling tool for medical diagnosis and research.[1]

Nitric oxide (NO) is a highly reactive free radical composed of one atom of nitrogen and one atom of oxygen. It is synthesized from substrate amino acid, l-arginine, by the enzyme nitric oxide synthase (NOS). It reacts with super oxides to form oxidant peroxynitrate which is responsible for cell injury.[2]

Lichen planus is a common mucocutaneous inflammatory disease affecting 0.05–1% of the world's population. Approximately half of the patients with cutaneous lichen planus have oral involvement. However, mucosal involvement can be the sole manifestation in up to 25% of the affected population.[3] Oral lichen planus (OLP) has a peak incidence in middle-aged patients and has a female predominance of

2:1.[4] Various etiological factors implicated in lichen planus are psychosomatic stress, dental materials, medications, viral infection (hepatitis C) and autoimmunity.[5] Recently, immunological imbalance has been considered in the etiology of lichen planus and literature reveals a possible correlation between NO level and OLP.[6]

The available literature reveals very little information in this regard. Hence, the present study was undertaken to estimate the levels of salivary NO in OLP, to ascertain its diagnostic as well as prognostic value.

Review of literature

Masaru Ohashi, Masayasu Iwase, and Masao Nagumo in 1999 measured the levels of NO in the saliva of 39 patients with oral mucosal diseases: 21 had OLP and 18 had recurrent aphthous ulceration (RAU). NO levels in the saliva of patients were significantly increased relative to those of healthy subjects.[7]

Sunita and Shanmugam in 2000 conducted a study to evaluate the levels of salivary NO in oral mucosal conditions. Twenty patients in each group, i.e. lichen planus, RAU, and control group, were included in the study. NO levels in the saliva of patients with lichen planus and RAU were significantly increased relative to those of healthy subjects.[8]

Ergun in 2011 conducted a study to assess oxidative stress and antioxidant profile in patients with OLP using serum and salivary samples and to compare these biomarkers in a group of healthy subjects. Twenty patients in each group, i.e. lichen planus and controls, were included in the study. Total antioxidant activity (TAA) and lipid peroxidation product malondialdehyde (MDA) in both serum and saliva were determined. Total antioxidant defense (TAA) was significantly lower in lichen planus than that in healthy subjects in their serum samples (P = 0.01). Salivary MDA levels were significantly higher in the OLP group compared with healthy subjects (P = 0.03).[9]

Aim

To estimate the level of salivary NO in patients with OLP and compare it with that of healthy subjects (controls).

Objectives

- To estimate salivary NO level in patients with OLP.
- To estimate salivary NO level in healthy individuals.
- To compare salivary NO levels in patients with OLP and healthy individuals.
- To assess the diagnostic and prognostic significance of salivary NO levels in patients with OLP.

METHODS

The study was carried out in the Department of Oral Medicine and Radiology, KLE VKI Institute of Dental Sciences, Belgaum. Sixty subjects were selected from the Out-patient Department and were divided into two groups: Group I, 30 subjects with clinically (WHO diagnostic criteria)[10] and histopathologically confirmed OLP and Group II, 30 healthy subjects free of any deleterious habits and periodontal disease (controls). Consent was obtained from all patients and volunteers.

Saliva samples were collected by asking the patients to rinse their mouth with water 5 min before the collection of the sample in any part of the day without being specific about time and food intake by the patient. Subjects were then asked to rinse their mouth with 5 ml of normal saline for 3 min. This was expectorated into a sterile beaker. The samples were refrigerated at 4°C without centrifugation and processed within 24 h. Salivary estimation of NO was done using Griess reaction with sulfanilic acid and naphthylenediamine in an acidic medium as per the method of Steuehr and Marletta in 1985.

Principle

The reduction of nitrate to nitrite is denoted by a color development when nitrate reacts with two reagents, sulfanilic acid and α-naphthylamine. The resulting color reaction is due to the formation of a diazonium compound, *p*-sulfonyl benzene-azo-α-naphthylamine (intensity of color is directly proportional to the concentration of salivary NO in the sample).

Composition of the Reagents

Reagent A

Ingredients

α-Naphthylamine 5 g and acetic acid (5 N) 30% 1000 ml.

Method of preparation

α-Naphthylamine is dissolved in less than 100 ml of 5 N acetic acid by gently heating 30 ml of glacial acetic acid. The solution is then transferred to a liter volumetric flask and is made up to 1000 ml with 5 N acetic acid. The solution is filtered through washed absorbent cotton and stored in a glass stoppered brown bottle.

Reagent B

Sulfanilic acid 8 mg (P-aminobenzene sulfanilic acid) and acetic acid (5 N) 30% 1000 ml.

Sulfanilic acid is dissolved in less than 1000 ml of 5 N acetic acid. The solution is then transferred to a liter volumetric flask and made up to 1000 ml with 5 N acetic acid.

0.1 ml of saliva was pipetted into the wells of flat-bottomed 96-well microtitration plate, followed by addition of 0.1 ml of freshly mixed equal parts of Griess reagents A and B. The plate was shaken for 10 min at room temperature, after which purple color developed in positive plates. The plates were read in a microplate reader at 570 nm with a reference value of 630 nm, and NO_2 concentration was determined from the calibration curve.

The data were collected and results were statistically analyzed using Student's "*t*" test.

RESULTS

All patients in our study belonged to the age group of 25–55 years. Among the study group patients, 11 patients (36.66%) were between the ages of 25 and 34 years, 9 patients (30.00%) were between 35 and 44 years, and 10 patients (33.33%) were between 45 and 55 years old. Similar findings were reported by Ali and Suresh, who quoted that lichen planus is a disease of adulthood, with age ranging from 30 to 70 years.[11]

Among the clinical types, majority of the patients had reticular lichen planus (27 patients), whereas 3 patients had erosive lichen planus. This was also in accordance with the studies conducted by Jensen *et al*., who stated that reticular lichen planus is the most common form of OLP.[12] The correlation of sodium nitrite concentration and optical density (OD) of standard solutions was determined. The range of OD for Group I was 0.11–0.60 and for Group II was 0.01–0.09 [Table 1, Graph 1]. There was statistically significant difference in the OD of sodium nitrite between the study and control groups.

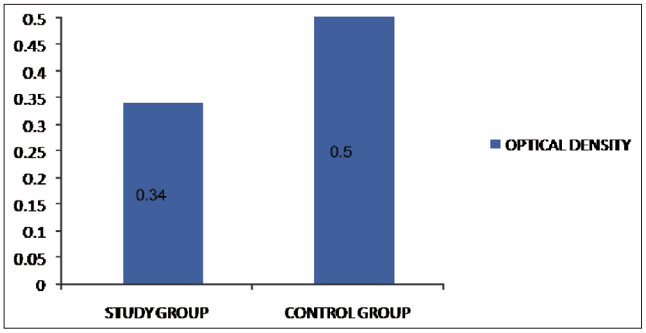
The range of NO for Group I was 20–90 μM and for Group II was 05–13 μM. Mean salivary NO of the study group (Mean ± SD) was 60.90 ± 21.86 and of the control group (Mean ± SD) was 07.20 ± 02.96 [Table 2, Graph 2]. There was statistically significant difference in the levels of salivary NO between the study and control groups.

DISCUSSION

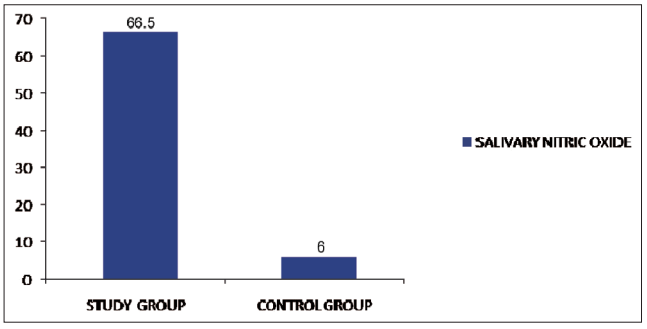
The enzyme NOS is capable of producing NO over a longer period of time by immunological mediators and cells including macrophages, T-cells, and natural killer cells. This study was aimed to estimate the level of salivary NO in patients with OLP and compare the levels with those of healthy individuals.

Table 1: Range of optical density (OD) in the study group and control group

Group	Range	Median
Study	0.11 - 0.60	0.34
Control	0.01- 0.09	0.50



Graph 1: Optical density of sodium nitrite among study and control groups



Graph 2: Salivary nitric oxide levels among study and control groups

Table 2: Range of salivary nitric oxide in the study group and control group

Group	Range	Median
Study	20 - 90	66.5
Control	05- 13	6

There were more males than females in the study group (1.3:1), which is contrary to the findings reported by Jensen *et al*., who found a female to male ratio of 1.5:1.[12] The male: Female ratio is purely a coincidental finding with no possible reason for the same.

The salivary NO levels in the study group ranged from 20 to 90 μM and that in the control group ranged from 5 to 13 μM. The values of NO in the study group were significantly raised, when compared with that of control group. This was similar to the findings reported by Sunitha *et al*., who concluded that the pathophysiology of OLP was associated with an increase in NO.[8]

Sessa described three isoforms of NOS, and according to him, type 3 or inducible NOS is found in oral mucosa, endothelial cells, and salivary gland tissue.[13] The possible cellular source of salivary NO is from nerve endings, salivary gland endothelial cells, or macrophages in response to oral bacterial products. It has been observed that there are psychological aspects to the etiology of OLP. Levels of hormones in saliva, such as cortisone, are reported to be high in patients with OLP.

Tsuchiya *et al.* have reported that psychological stress causes NO release in correlation with increase of neural NOS activity.[14] This is the explanation for the elevated NO production in patients with OLP. A careful observation into the histopathologic picture of lichen planus reveals the presence of hyperkeratosis, acanthosis, saw-tooth rete ridges, degeneration of basal cell layer, and a well-defined band-like inflammatory cell infiltrate consisting mainly of lymphocytes including CD4 and CD8 cells in the subepithelial connective tissue.[15]

Yamanoto *et al.* in their *in vitro* studies observed that T lymphocytes from lichen planus tissue produced increased levels of interleukin (IL)-6 and granulocyte macrophage colony stimulating factors (GM-CSF) and can be stimulated to produce more tumor necrosis factor (TNF)-α by IL-3, IL-6, and GM-CSF.[16] *In vitro* studies have shown that keratinocytes from OLP lesions produce interferon (IFN)-a, IL-6, and TNF-α in response to IL-3. Thus, these keratinocytes have the potential to produce large amount of cytokines capable of activating infiltrating T cells. These cytokines, interleukins, and TNF are capable of activating T cells, and thus contribute to the release of NO.

Ohashi *et al.* noted the effects of NO on cultured cells using NO donating agents and found that it caused severe damage to fibroblasts, keratinocytes, and oral epithelial cells *in vitro*.[7] Volk *et al.* noted the cytotoxic potential of NO against various cells.[17] Moncada *et al.* opined that when generated in excess, NO is a key mediator of cell damage, tissue injury, and organ failure.[18]

Das *et al.* have indicated that free radicals including NO may play an important role in ulceration induced by several kinds of stress.[19] Therefore, NO is considered to cause erosion and ulceration as a consequence of cell damage. Thus, it is proposed that free radicals including NO represent one route of pathogenesis and that excess of salivary NO may have pathophysiological implications for erosive and ulcerative lesions in OLP.

REFERENCES

1. Brosky ME. The Role of Saliva in Oral Health: Strategies for prevention and Management of Xerostomia. J Support Oncol 2007;5:215-25.
2. Dagigh F, Borghaei RC, Borghaei RD, Bee JH. Human gingival fibroblasts produce nitric oxide in response to proinflammatory cytokines. J Clin Periodontol 2002;73:392-400.3. Mollaoglu N. Oral lichen planus: A review. Br J Oral Maxillofac Surg 2000;38:370-7.
4. Setterfield JF, Black MM, Challacombe SJ. The management of oral lichen planus. Clin Exp Dermatol 2000;25:176-82.
5. Eisen D. The evaluation of cutaneous, genital, scalp, nail, eosophageal and ocular involvement in patients with oral lichen planus. Oral Surg Oral Med oral Pathol Oral Radiol Endod 1999;88:431-6.
6. Carrozzo M, Gandolfo S, Lodi G, Carbone M, Garzino-Demo P, Carbonero C, *et al*. Oral lichen planus in patients infected or non-infected with hepatitis C virus: The role of autoimmunity. J Oral Pathol Med 1999;28:16-9.
7. Ohashi M, Iwase M, Nagumo M. Elevated production of salivary nitric oxide in oral mucosal diseases. J Oral

Pathol Med 1999;28:355-9.
8. Sunitha M, Shanmugam S. Evaluation of salivary nitric oxide levels in oral mucosal diseases: A controlled clinical trial. Indian J Dent Res 2006;17:117-20.
9. Ergun S, Trosala SC, Wamakulasuniya S, Onal AE, Ofluoglu D, Guven Y, *et al*. Evaluation of oxidative stress and antioxidant profile in patients with oral lichen planus. J Oral Pathol Med 2011;40:286-93.
10. Rad M, Hashemipoor MA, Mojtahedi A, Zarei MR, Chamani G, Kakoei S, *et al*. Correlation between clinical and histopathologic diagnosis of oral lichen planus based on modified WHO criteria. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;107:796-800.
11. Ali AA, Suresh CS. Oral lichen planus in relation to transaminase levels and hepatitis C virus. J Oral Pathol Med 2007;36:604-8.
12. Neumann- Jensen B, Holmstrup P, Pindborg JJ. Smoking habits of 611 patients with oral lichen planus. J Oral Surg 1977;43:410-5.
13. Sessa WC, Prichard K, Seyedi N, Wang J. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ Res 1994;74:349-53.
14. Tsuchiya T, Kishimoto J, Koyama J, Ozawa T. Modulatory effect of L-NAME, a specific nitric oxide synthase (NOS) inhibitor or stress-induced changes in plasma adreno-corticotropic hormone and corticosterone levels in rats. Brain Res 1997;776:68-74.
15. Matthews JB, Basu MK, Potis AJC. Macrophages in oral lichen planus. J Oral Pathol 1985;14:553-8.
16. Yamamoto T, Osaki T, Yoned K, Utea E. Cytokine production by keratinocytes and mononuclear infiltration in oral lichen planus, J Oral Pathol Med 1994;23:309-15.
17. Volk T, Ioamridis I, Hensel M, Degroot H. Endothelial damage induced by nitric oxide synergism with reactive oxygen species. Biochem Biophys Res Commun 1995;213:1196-203.
18. Monacada S, Palmer RMJ, Higgs EA. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-42.
19. Das D, Bandyopadhyay D, Bhattacharjee M, Banerjee RK. Hydroxyl radical is the major causative factor in stress induced gastric ulceration. Free Radic Biol Med 1997;23:8-18.

Source of Support: Nil **Conflict of Interest:** None declared.