Caspian Journal of Dental Research
e-ISSN: 2322-2395 p-ISSN: 2251-9890
Babol University Of Medical Sciences

Evaluation of the relationship between nitric oxide and candida albicans-associated denture stomatitis: A cross-sectional study

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Article type

Research Paper

Received: 27 May 2023
Revised: 26 Jul 2023
Accepted: 16 Aug 2023
Pub. online: 11 Sept 2023

ABSTRACT

Introduction: Candida albicans (C. Albicans) is an opportunistic microorganism of the normal flora that can cause infection in the oral mucosa. Nitric oxide (NO) is a free radical produced by macrophages and is highly associated with antifungal activities. The aim of this study was to evaluate salivary nitric oxide levels in patients with and without Candida Albicans-associated denture stomatitis.

Materials & Methods: In this cross-sectional study, 40 edentulous patients using dentures were divided into two groups: patients with and without denture stomatitis (DS). Before laboratory detection of candida, an oral medicine specialist clinically confirmed the presence of DS. Saliva samples were collected by spitting method, and the Griess method measured NO. Chi-square and Mann-Whitney tests were used for statistical analysis. The level of significance was considered 0.05.

Results: The present study showed that the NO level was significantly higher in patients with DS than in patients without DS (P-value=0.002). In this study, the mean NO level in patients with DS was 166.5485±43.538 µM, while that was 118.0585±47.617 µM for patients without DS.

Conclusion: NO concentration in patients' saliva can be associated with C. Albicans infection in the oral cavity. In the presence of Candida, the level of NO increases, and it seems that this increase is a kind of defense response to the presence of fungal infections.

Keywords: Candidiasis, Candida Albicans, Denture Stomatitis, Nitric Oxide, Saliva

Cite this article: Shoorgashti R, Khalilirad M, Haji Molla Hoseini M, Lesan S. Evaluation of the relationship between nitric oxide and candida albicans-associated denture stomatitis: A cross-sectional study. Caspian J Dent Res 2023; 12: 82-8.

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Introduction

Nitric oxide (NO) is a free radical on which several physiological processes rely. [1, 2] NO plays a vital role in various physiological processes, including angiogenesis, anti-apoptosis, anti-inflammation, antithrombosis, vasodilation, and immune response. This short-lived, biologically active component is produced as a part of the innate immune system. In mammals, NO acts as a signaling molecule and is produced by Macrophages. [3-6] Reactive nitrogen species (RNS) formed by NO exhibit broad-spectrum antibacterial and antifungal properties. [1,2] By interacting with and inactivating bacterial deoxyribonucleic acid (DNA), proteins, and enzymes, RNS molecules can cause bacterial death. In contrast to conventional antibacterial drugs, NO has unique antibacterial properties that significantly reduce the occurrence of bacterial resistance. So, various diseases can be treated with NO. [2-5]

The condition of denture stomatitis (DS) is inflammation of the oral mucous membrane resulting from a removable, partial, or complete prosthesis. In patients with DS, denture-bearing mucosa commonly exhibits inflammation, erythema, and edema, which can be asymptomatic or accompanied by pain or burning sensation. [6-9] DS is a high-prevalence problem; 20 to 67% of patients with removable prosthetic appliances suffer from DS. [10] Some DS-related factors include denture-wearing patterns, poor hygiene, systemic diseases, immune system disorders, and bacterial and yeast infections. [6, 9, 10] Candida Albicans (C. Albicans) is known as the most associated fungi with DS. It is an opportunistic microorganism of the normal flora and the most common oral pathology in the elderly. [9, 11, 12] There are controversial results from previous studies on the relationship between DS associated with C. Albicans and NO level. Gasparoto [13] found that the level of NO increased in both groups of patients with DS and without DS. Madariaga-Venegas [12] stated that NO had no effect on fungal colonization in agar media. However, Casaroto [14] showed an increase in the level of NO in the presence of candida infections. The aim of the current study was to evaluate salivary nitric oxide levels in patients with and without Candida Albicans-associated denture stomatitis.

Materials & Methods

The methodology of this study was approved by the ethical committee under the ethical code of IR.IAU.DENTAL.REC.1398.013.

Participants and sampling of saliva: In this cross-sectional (descriptive-comparative) study, 40 participants were selected in 3 months (January-March 2019). All participants were evaluated based on inclusion and exclusion criteria. Edentulous patients with at least a single maxillary complete denture and no history of alcoholism, diabetes, and xerostomia treatments, were included in this study. Since antibiotics could be a modifiable iatrogenic risk factor for the most common human fungal infection and consumption of antifungals can influence the colonization of candida [15, 16], patients taking antifungals or antibiotics at the time of the study were excluded. The sample size of this study was calculated as 20 participants in each group based on the conducted study by Gasparoto [13] (Power = 80%, the mean difference = 0.1, and the level of statistical significance (α) = 0.05). 20 patients presenting Candida-associated DS and 20 without signs of DS (aged 40 to 80) were selected. An oral medicine specialist clinically confirmed the presence of DS, and its classification was performed based on Newton. [17, 18] In this classification system, DS has three subtypes; Type 1: localized or pinpoint inflammation; type 2:

[DOR: 20.1001.1.22519890.2023.12.2.4.9]
[DOI: 10.22088/cjdr.12.2.82]

generalized erythematous that partially or completely affects the mucosa covered by dentures; type 3: nodular or papillary hyperplasia that usually affects the central part of hard palate or alveolar ridge. [19]

Microbiological testing was conducted by taking samples from the hard palate, underlying dentures, and the mucosa-bearing surfaces of dentures with sterile swabs. A selective medium was used to cultivate the samples (CHROMagar Candida, Taligene Pars, Isfahan, Iran), and Gram staining was performed on them. Based on the automated reading of ID32C-inoculated test kits (bioMerieux, Basingstoke, UK), the isolates were identified. [13, 18] After obtaining an informed contest, participants were asked not to drink, eat, chew gum or mints, and brush their teeth for at least 2 hours before saliva sampling. Non-stimulation saliva samples were collected by spitting method in sterilized bottles for at least 1.5 mL between 7 and 9 am.

Nitric Oxide evaluation: NO has an extremely short half-life (3-4 seconds), and measurement of its concentration is difficult. [2] In this study, the Griess method was used. In this method, the level of nitrite as an indicator for NO was measured. In the first step, by reacting sulfanilamide with dinitrogen trioxide [N2O3], diazobenzenesulfonic acid (an N2O3 derivative) is formed. Then, this intermediate product reacts to N- (1-naphthyl) ethylenediamine (NED), and as a result of this reaction, azo-α-aminonaphthalene parabenzene sulfonic acid, which is a purple-colored substance is produced. This purple product can be measured at 520-550nm wavelength. [20]

Preparation of Griess reagent and standardized the concentrations: To prepare the Griess reagent, we need two solutions with specific concentrations, namely 1% sulfanilamide and 0.1% NED. To prepare 50 milliliters [mL] of 1% sulfanilamide solution, 0.5 grams [g] of this substance was dissolved in 50 mL of 5% phosphoric acid; and to prepare 50 mL of 0.1% NED, 0.05 g of that was dissolved in 50 mL of deionized water. Both solutions can be stored in a refrigerator, away from light, for about two months. [21]

To prepare the standard dilution, sodium nitrite with a molecular weight of 69 Dalton was used. 0.0069 g of sodium nitrite powder was weighed with a Digital analytical scale (AXIS , Warsaw, Poland) and dissolved in deionized water to obtain a 10 mL solution with a concentration of 10^4 micromolar [μM]. Then, 100 microliters [μL] of this solution were added to deionized water to obtain 1mL solution with a concentration of 10^3 μM. This stage was repeated one more time until the concentration of the solution reached 100 μM. After that, 100 μM-concentrated solutions were used in serial dilution, which was applied using 98-well plates.

100 μL of 100 μM solution was poured into A1 to A3 wells and considered the standard concentration. Then, 50 μL of deionized water was poured in triplicate in wells B to G. After that, 50 μL of the standard solution in wells A1 to A3 was added to deionized water in B1 to B3, then the solution in B1 to B3 wells was added to C1 to C3. In this way, serial dilution was performed. It should be noted that no standard solution was added to H1 to H3 wells, which was zero concentration.

Nitric oxide measurement: 50μL of samples were added in triplicate to wells. After that, 50 μL of Sulfanilamide solution was added to all wells (standards, samples, and blanks). After incubation for 5 minutes [min] at room temperature, 50 μL of NED solution was added to the wells and incubated for 10 min in a dark place at room temperature. Because Sulfanilamide and NED solution was kept in the refrigerator, they were left to stand at room temperature. When a spectrum of purple color was formed, the optical absorption of each color was read at an appropriate wavelength using the ELISA Reader (Biotek, Winooski, USA). [13, 22] It should be noted that although the colors are stable for 30 min, they

[DOR: 20.1001.1.22519890.2023.12.2.4.9]
[DOI: 10.22088/cjdr.12.2.82]

should be read at 520 to 550nm wavelength after 10 min. A standard curve was designed based on the results of the ELISA Reader. Then, the concentration of NO was calculated using this curve and reported in μM.

Statistical analysis: All data were transferred to SPSS software, and the median level of NO was compared between 20 patients with C. Albicans-associated denture stomatitis and 20 patients without it using the Mann- Whitney U test. The Chi-square test was performed to compare the age and the years using dentures in 2 groups. In addition, the Bivariant Correlation tests were used to determine any correlation between the NO level and either age of participants or the number of years they were using dentures. The level of significance was considered ≤ 0.05.

Results

Among 40 edentulous participants were selected based on inclusion and exclusion criteria during 3 months. They were divided into two groups: 1. Patients with DS and 2. Patients without DS. The number of females and males participating in the study was similar in both groups (4 females and 16 males). Each group included 3 smokers (15%) and one participant with high blood pressure (5%). No participants were taking medication during the study. In the DS group, 4 participants had type 2 DS (20%), and 16 had type 1 DS. Table 1. shows the concentration of NO in two groups:

Table 1. The level of NO in DS and non-DS groups

Groups	Sample size	The lowest concentration of NO (μM)	The highest concentration of NO (μM)	Mean ±SD concentration of NO (μM)	P-value *
DS	20	87.90	231.79	166.548 ±43.538	0.002
Non-DS	20	68.76	228.50	118.058 ±47.617	

DS: Denture stomatitis
Non-DS: Non-denture stomatitis
*: Mann- Whitney U test

Based on the Mann-U-Whitney test, the NO level in the saliva of patients with DS was significantly higher than in the non-DS group (P-value = 0.002). The mean age and years that patients have been using dentures are shown in Table 2. Based on the Chi-square test, there was no significant difference between the two groups regarding the age of participants (P-value=0.37). Also, no considerable difference was seen in the years of using dentures in the two groups (P-value=0.22) (Table 2). Based on the Bivariant Correlation tests, there was no statistically significant correlation between the age of patients and the level of NO in both DS and Non-DS groups (P value=0.68 and P value=0.23, respectively). Also, no statistically significant correlation between years of using dentures and the NO level was found in the two groups (P value= 0.43 and P value=0.16, respectively).

Table 2. The mean ±SD of age and the years of patients using dentures

	Groups	Sample size	Mean ±SD	P-value
Age	DS	20	58.8 ±8.62	0.37
	Non-DS	20	60.65 ±8.83	
Years of using dentures	DS	20	8.85 ±3.16	0.22
	Non-DS	20	8.4 ±3.01	

DS: Denture stomatitis, Non-DS: Non-denture stomatitis, Min.: Minimum, Max.: Maximum

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Discussion

This study's results revealed that NO concentration was significantly higher in DS patients than in non-DS patients. The NO mean concentration was about 50 µM higher in the DS group than in the non-DS (166.54±43.53 and 118.05±47.61, respectively). Similar findings were discovered in multiple studies. [13, 23-26] In Gasparato's study, elderly DS patients had elevated levels of NO, IL-6, CCL3, and TGF-β. [13] Liao's study found that patients produced NO in response to C. Albicans-related DS. [23] Casaroto's study showed that infection with C. Albicans has the potential to elevate the production of NO. [14] The biofilm of Candida can grow and proliferate in the fitting surface of removable prosthetic appliances. [27] The reduced space beneath dentures creates an environment that is conducive to Candida pathogenesis. Even without direct contact, C. Albicans can harm the human palate epithelium. To combat the invasion of fungi, human epithelial cells produce NO and hBD-2 antimicrobial peptides. [14, 28]

However, Hillestand found no difference in NO and nitrite levels between patients infected and not infected with C. albicans, which may be due to differences in measuring NO levels and the inclusion of cancer patients in their study. [18] Poor hygiene and improper denture care can lead to inflamed denture-bearing tissues. C. Albicans is known to play a significant role in this multifactorial disease known as DS. [29-31] NO and LL-4 was found to inhibit C. Albicans growth. Elahi found that the increased NO production can lead to reduced yeast growth in mice, and LL-4 is associated with C. Albicans protection in patients. [24] The NO production growth in DS patients is also related to the neutrophils' apoptosis. Although inducible nitric oxide synthase (iNOS) is included with neutrophils enrollment to the location of contamination, the part of NO actuating apoptosis is, to a great extent, known. [13]

NO seems to control the colonization of C. Albicans on the surface of the oral mucosa and can also increase the macrophages' protection activities. The rapid increase of NO in mice was related to fungi infection, and their resistance to this infection was known to be correlated to this substance. The mechanisms by which NO reduces the growth of C. Albicans have been unknown. However, a decrease in PH due to NO production can be one of the reasons behind that. [24] Other than that, C. Albicans uses several mechanisms to counter nitrosative stress. The highly represented gene in C. Albicans is YHB1 which can code protein enzymes breaking down NO. This microorganism uses flavohemoglobin-mediated detoxification, antioxidant-mediated scavenging, and activation of repair mechanisms to protect itself from NO. Many useful antifungal drugs have been reported to become resistant to fungi, so new antifungal drugs with novel mechanisms must be developed. Considering NO effects on fungus, it is possible to develop an innovative therapy that uses a stable NO-releasing nanoparticle platform unlikely to evolve resistance in fungi. [23]

Despite the strengths of the study, its findings were limited by the small sample size of only 40 participants, most of whom were male. For future studies, it would be beneficial to include a larger and more diverse sample size, including more female subjects to explore the potential impact of sex hormones on the oral cavity environment.

Conclusion

In the current study, the NO level in C. Albicans- associated DS patients were significantly higher than that for Candida A-free patients. So, it seems that NO concentration in patients' saliva can be associated with C. Albicans infection in the oral cavity, which is an immune response to fungal infection.

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Considering Candida's increasing number of antifungal-resistant strains, NO-based medication can be a beneficial alternative. However, more animal and clinical trial studies must be conducted to clarify the merits and demerits of NO-based drugs. The ongoing study can be a hint for designing further trial studies.

Acknowledgments

The authors would like to thank the participants for their cooperation.

Funding

This study was a research project (No: 260_23) supported by Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.

Conflicts of Interest

All authors declare no conflict of interest.

Author's Contribution

Simin Lesan and Mostafa Haji Molla Hoseini developed the original idea and the protocol. They supervised the project and critically revised the manuscript for important intellectual content. Reyhaneh Shoorgashti abstracted, interpreted, and analyzed data and wrote the manuscript. Mahsa Khalilirad contributed to the abstracted data and prepared the manuscript.

References

1. Collette JR, Zhou H, Lorenz MC. Candida albicans Suppresses Nitric Oxide Generation from Macrophages via a secreted Molecule. PLoS One. 2014;9:e96203.
2. Qian H, Ye Z, Pi L, Ao J. Roles and current applications of S-nitrosoglutathione in anti-infective biomaterials. Mater Today Bio. 2022;16:100419.
3. Mao Y-J, Wu J-B, Yang Z-Q, Zhang Y-H, Huang Z-J. Nitric oxide donating anti-glaucoma drugs: advances and prospects. Chin J Nat Med. 2020;18:275-83.
4. Pinto RV, Carvalho S, Antunes F, Pires J, Pinto ML. Emerging Nitric Oxide and Hydrogen Sulfide Releasing Carriers for Skin Wound Healing Therapy. ChemMedChem. 2022;17:e202100429.
5. He Q, Qu M, Xu C, Shi W, Hussain M, Jin G, et al. The emerging roles of nitric oxide in ferroptosis and pyroptosis of tumor cells. Life Sci. 2022;290:120257.
6. Sardari F, Khalili P, Hakimi H, Mahmoudaghaei S, Abedi P. The prevalence of denture stomatitis in cigarette and hookah smokers and opium addicts: findings from Rafsanjan Cohort Study. BMC Oral Health. 2021;21:455.
7. Gad MM, Fouda SM. Current perspectives and the future of Candida albicans-associated denture stomatitis treatment. Dent Med Probl. 2020;57:95-102.
8. Moraes GS, Albach T, Sugio CY, de Oliveira FB, Neppelenbroek KH, Urban VM. Experimental animal models for denture stomatitis: A methodological review. Lab Anim. 2022;56:331-43.
9. Figueiral MH, Azul A, Pinto E, Fonseca PA, Branco FM, Scully C. Denture-related stomatitis: identification of aetiological and predisposing factors - a large cohort. J Oral Rehabil. 2007;34:448-55.

[DOR: 20.1001.1.22519890.2023.12.2.4.9]
[DOI: 10.22088/cjdr.12.2.82]

10. Sartawi SY, Abu-Hammad SA, Salim N, Al-Omoush S. Denture Stomatitis Revisited: A Summary of Systematic Reviews in the Past Decade and Two Case Reports of Papillary Hyperplasia of Unusual Locations. Int J Dent. 2021;2021:7338143.
11. Alvendal C, Ehrström S, Brauner A, Lundberg JO, Bohm-Starke N. Elevated nitric oxide in recurrent vulvovaginal candidiasis - association with clinical findings. Acta Obstet Gynecol Scand. 2017;96:295-301.
12. Madariaga-Venegas F, Fernández-Soto R, Duarte LF, Suarez N, Delgadillo D, Jara JA, et al. Characterization of a novel antibiofilm effect of nitric oxide-releasing aspirin (NCX-4040) on Candida albicans isolates from denture stomatitis patients. PLoS One. 2017;12:e0176755.
13. Gasparoto TH, Sipert CR, de Oliveira CE, Porto VC, Santos CF, Campanelli AP, et al. Salivary immunity in elderly individuals presented with Candida-related denture stomatitis. Gerodontology. 2012;29:e331-9.
14. Casaroto AR, da Silva RA, Salmeron S, Rezende MLR, Dionísio TJ, Santos CFD, et al. Candida albicans-Cell Interactions Activate Innate Immune Defense in Human Palate Epithelial Primary Cells via Nitric Oxide (NO) and β-Defensin 2 (hBD-2). Cell. 2019;8:707.
15. Drummond RA, Desai JV, Ricotta EE, Swamydas M, Deming C, Conlan S, et al. Long-term antibiotic exposure promotes mortality after systemic fungal infection by driving lymphocyte dysfunction and systemic escape of commensal bacteria. Cell Host Microbe. 2022;30:1020-33.e6.
16. Dey R, Mukherjee S, Barman S, Haldar J. Macromolecular Nanotherapeutics and Antibiotic Adjuvants to Tackle Bacterial and Fungal Infections. Macromol Biosci. 2021;21:e2100182.
17. Barros S, Al-Tarawneh S, Bencharit S, Loewy Z, Gendreau L, Offenbacher S. Salivary cytokines and levels in denture stomatitis: An exploratory case-control study. Open J stomatol. 2012;2:326-33.
18. Hillestad J, Bordin P, Bockman OC, Mortensen B, Bjornland T, Olsen I. Relationship between nitrate\nitrite concentration in saliva and oral candidosis. Microb Ecol Health Dis. 2005;17:83-7.
19. Coronado-Castellote L, Jimenez –Soriano Y. Clinical and microbiological diagnosis of oral candidiasis. . J Clin Exp Dent. 2013;5:e279.
20. Goshi E, Zhou G, He Q. Nitric oxide detection methods in vitro and in vivo. Med Gas Res. 2019;9:192-207.
21. Hakobyan L, Monforte-Gómez B, Moliner-Martínez Y, Molins-Legua C, Campíns-Falcó P. Improving Sustainability of the Griess Reaction by Reagent Stabilization on PDMS Membranes and ZnNPs as Reductor of Nitrates: Application to Different Water Samples. Polymers (Basel). 2022;14:464.
22. Giustarini D, Rossi R, Milzani A, Dalle-Donne I. Nitrite and nitrate measurement by Griess reagent in human plasma: evaluation of interferences and standardization. Methods Enzymol. 2008;440:361-80.
23. Liao Z, Yan Y, Dong H, Zhu Z, Jiang Y, Cao Y. Endogenous nitric oxide accumulation is involved in the antifungal activity of Shikonin against Candida albicans. Emerg Microbes Infect. 2016;5:1-6.
24. Elahi S, Pang G, Ashman RB, Clancy R. Nitric oxide-enhanced resistance to oral candidiasis. Immunology. 2001;104:447-54.
25. Privett BJ NS, Schoenfisch MH. Efficacy of surface-generated nitric oxide agaibst Candida albicans and biofilm formation. Biofouling. 2010;26:973-83.
26. Liao Z, Zhu Z, Li L, Wang L, Wang H, Jiang Y, et al. Metabonomics on Candida albicans indicate the excessive H3K56ac is involved in the antifungal activity of Shikonin. Emerg Microbes Infect. 2019;8:1243-53.
27. Bajunaid SO. How Effective Are Antimicrobial Agents on Preventing the Adhesion of Candida albicans to Denture Base Acrylic Resin Materials? A Systematic Review. Polymers (Basel). 2022;14:908.
28. Le Bars P, Jordana F, Kouadio A. Denture Plaque Management of Denture-Related Stomatitis. Dent Adv Res. 2021;6.
29. Galvan R, McBride M, Korioth TV, Garcia-Godoy F. Denture Hygiene as It Relates to Denture Stomatitis: A Review. Compend Contin Educ Dent. 2021;42:e1-e4.
30. Hannah VE, O'Donnell L, Robertson D, Ramage G. Denture Stomatitis: Causes, Cures and Prevention. Prim Dent J. 2017;6:46-51.
31. Contaldo M, Romano A, Mascitti M, Fiori F, Della Vella F, Serpico R, et al. Association between denture stomatitis, candida species and diabetic status. J Biol Regul Homeost Agents. 2019;33:35-41.