

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jpfa



Correlation between nitric oxide concentration ($NO_2 + NO_3$) in saliva and CD4+ count in HIV infected adult individuals of Mangalore, Karnataka, India



Mithra N. Hegde [a,*], *Shilpa S. Shetty* [b], *Amit Malhotra* [a], *Nidarsh D. Hegde* [c]

[a] *Department of Conservative Dentistry and Endodontics, A.B. Shetty Memorial Institute of Dental Sciences, Deralakatte, Mangalore, Karnataka, India*

[b] *Nitte Education Centre for Science Education and Research, K.S. Hegde Medical Academy, Deralakatte, Mangalore, Karnataka, India*

[c] *Department of Oral and Maxillofacial Surgery, A.B. Shetty Memorial Institute of Dental Sciences, Deralakatte, Mangalore, Karnataka, India*

Keywords:
Nitric Oxide
Saliva
Dental Caries
HIV

ABSTRACT

Aims: To investigate the correlation between nitric oxide concentration ($NO_2 + NO_3$) in saliva and CD4+ count in HIV infected individuals.

Study design: A cross-sectional study was carried out among 60 proven HIV-positive patients selected randomly from the ART centres.

Methodology: Two groups – Control ($n = 30$) and study group ($n = 60$). The study group was divided into Group I (CD4+ count >200/cu.mm) and Group II (CD4+ count<200/cu.mm). Unstimulated saliva was collected and nitric oxide (NO_3+ NO_3) was estimated by Griess method. Statistical analysis was performed using One-way ANOVA and Tukey's Multiple Comparison Test.

Results: The mean level of nitric oxide ($NO_2 + NO_3$) in control group was 43.95 ± 3.64 and in Group I with CD4 >200/cu.mm and Group II with CD4 <200/cu.mm was 51.98 ± 4.08 and 59.78 ± 4.18 respectively. 'p' value was statistically significant ($P < 0.0001$). The mean difference between Control and Group I was −7.937 ($P < 0.001$), Control and Group II was −15.80 ($P < 0.001$) and Group I and Group II was −7.867 ($P < 0.001$) from Tukey's Multiple Comparison Test.

Conclusions: From our study it is now evident that salivary nitric oxide ($NO_2 + NO_3$) can serve as an indicator of HIV replication and saliva shows a strong potential to perform tests that are done currently in blood.

Copyright © 2014, Pierre Fauchard Academy (India Section). Publishing Services by Reed Elsevier India Pvt. Ltd. All rights reserved.

* *Corresponding author.*
E-mail address: drhegdedentist@gmail.com (M.N. Hegde).

http://dx.doi.org/10.1016/j.jpfa.2014.07.004

0970-2199/Copyright © 2014, Pierre Fauchard Academy (India Section). Publishing Services by Reed Elsevier India Pvt. Ltd. All rights reserved.

1. Introduction

HIV infection is a condition caused by the human immunodeficiency virus (HIV). The condition gradually destroys the immune system, which makes it harder for the body to fight infections.[1]

Nitric oxide (NO) is an important biologically active molecule that plays a key part in host defence against bacteria, protozoa, and tumour cells. NO has antiviral effects against several DNA viruses, such as murine poxvirus, herpes simplex virus, and Epstein–Barr virus, and some RNA viruses, such as coxsackievirus. NO is of particular interest among free radicals. This molecule is generated by nitric oxide synthase (NOS) from L-arginine and rapidly reacts in vivo with oxygen to form its two stable end products, nitrite and nitrate.[2–4] Three distinct isoforms of NOS have been described. Two are constitutive and mainly found in endothelial and neuronal cells the third, inducible NOS (iNOS), was originally described in murine macrophage. Numerous physiological functions are NO mediated and known to be implicated in several immunological disorders.[5]

The production of NO, in some cases during infectious diseases may also be deleterious which is particularly true in HIV infection, where NO may contribute to AIDS pathogenesis. Significant increases in nitrite and nitrate (the two stable end products of NO) concentrations were evidenced in peripheral blood mononuclear cells (PBMC), polymorphonuclear leukocytes, and sera of patients with AIDS, especially in individuals with neurological disorders and pulmonary disease caused by intracellular opportunistic pathogens.[6] Overproduction of NO has been noted in several studies both invitro and in vivo in the presence of HIV-1 infection. Furthermore, increased NO production may contribute directly to the pathogenesis of HIV-1-associated dementia.[7]

Saliva testing is an increasing area of research with implications for basic and clinical purposes as this fluid is easy to manipulate and collect. The use of saliva has provided a substantial addition to the diagnostic armamentarium as an investigative tool for disease processes and disorders recently.[8] The full power and potential of saliva in medical applications however was only recently recognized when saliva was shown to reflect the spectrum of health and disease states and to offer distinctive advantages over serum.[9,10] Today, a growing number of proof-of-principle assays have been established using saliva to monitor diseases or bodily conditions such as HIV infection.[11] The purpose of this study was to investigate the correlation between nitric oxide concentration ($NO_2 + NO_3$) in saliva and CD4+ count in HIV infected individuals.

2. Materials and methods

Ethical clearance was obtained from the Institutional Ethical Committee. 200 patients were screened, out of which 60 proven HIV-positive patients showing positive ELISA and Western blot test, without any concomitant systemic disease (other systemic diseases are known to cause changes in antioxidant levels) were selected from ART Centre, Wenlock Hospital, Mangalore, A.B. Shetty Memorial Institute of Dental Sciences, Deralakatte, Mangalore, K.S. Hegde Medical Academy (O.P.D. of Medical College), Deralakatte, Mangalore. CD4+ counts were determined using complete blood count and flow cytometry.

Subjects who satisfied the inclusion criteria were given the complete information regarding the nature of study, their participatory role with respect to physical examination and implication of the study. They were also asked to sign an informed consent form before their recruitment into study.

The case history was recorded in a prescribed format attached with the original protocol and oral examination was done.

2.1. Groups

Control Group: A total of 30 subjects, were taken in control group.

Study Groups: A total of 60 proven HIV subjects, who satisfied the inclusion and exclusion criteria were taken. These were further divided into two groups:

Group I: 30 subjects with CD4+ lymphocyte count >200/cu.mm which suggests early HIV infection.

Group II: 30 subjects with CD4+ lymphocyte count<200/cu.mm signifies the condition known as acquired immune deficiency syndrome.

2.2. Collection of saliva

Collection of saliva was done in the noon time before food to maintain the uniformity of the composition. Unstimulated saliva was collected from a patient who is not involved in any masticatory function in the last two hours prior to saliva collection and is seated in an ordinary chair and not on any dental/operatory chair to avoid anxiety. The saliva was allowed to accumulate in patient's mouth for 2 min and was aspirated directly from the floor of the mouth. Each sample was estimated for nitric oxide concentration ($NO_2 + NO_3$).

2.3. Estimation of nitric oxide concentration by Griess reaction method

Nitric oxide concentration was measured as total nitrates and nitrites ($NO_2 + NO_3$) by the Griess reaction method. Absorbance is read at 550 nm. Concentration is determined using standard graph.

2.4. Statistical analysis

One-way ANOVA was used to correlate between total antioxidant level and dental caries between the control and the two study groups. Tukey's Multiple Comparison Test was performed for intergroup comparison. A 'p' value of 0.05 or less was considered significant. Results are presented as mean ± standard deviation (X ± SD).

3. Results

The mean level of nitric oxide ($NO_2 + NO_3$) concentration in control group was 43.95 ± 3.64 and in Group I with CD4

Table 1 – Correlation between nitric oxide concentration ($NO_2 + NO_3$) in saliva and CD4+ count in HIV infected individual.

Parameters	Control group ($n = 30$) μM/L (mean ± SD)	Study group ($n = 30$) μM/L (mean ± SD)		'P' value
Nitric oxide (NO2 + NO3)	43.95 ± 3.64	Group I CD4>200/cu.mm 51.98 ± 4.05	Group II CD4<200/cu.mm 59.78 ± 4.18	P < 0.0001

*P < 0.05 is statistically significant. Statistical comparison were performed by One-way ANOVA. Data expressed as Mean ± SD.

>200/cu.mm and Group II with CD4 <200/cu.mm was 51.98 ± 4.08 and 59.78 ± 4.18 respectively. '*p*' value was statistically significant ($P < 0.0001$) (Table 1, Fig. 1). Tukey's Multiple Comparison Test was also performed to compare the statistical significance between groups. The mean difference between Control and Group I was −7.937 ($P < 0.001$), Control and Group II was −15.80 ($P < 0.001$) and Group I and Group II was −7.867 ($P < 0.001$) (Table 2).

4. Discussion

Nitric oxide (NO), an inorganic free-radical gas which has been found to have several fundamental roles in the biology of higher organisms whose production is catalyzed in vivo by a series of three independent enzymes inappropriately called synthases.[12] The role of nitric oxide (NO) is multidimensional. It functions as an intracellular messenger and is also implicated as a deleterious agent in various pathophysiological conditions including cancer, inflammatory conditions and autoimmune diseases.[13–15] Nitric oxide (NO) is an omnipresent intercellular messenger in all vertebrates, modulating blood flow, thrombosis, and neural activity and also in fighting infection.[16]

Correlations of nitrite and nitrate with the immune activation markers (sTNFR 55 and TNFR 75) and neopterin in HIV-1- infected patients suggest that endogenous cytokines, like TNF-a, could activate inflammatory cells. Nitric oxide may in turn contribute to the immunopathogenesis of HIV disease both by enhancing HIV replication and by direct effects on target tissues, such as the brain and lung by the increased production of cytokines.[5] The significance of NO production in HIV infection has been well established. Nitrite and citrulline

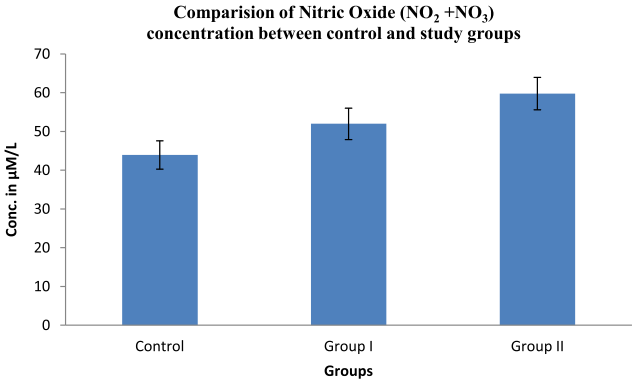


Fig. 1 – Graphical representation of correlation between nitric oxide ($NO_3 + NO_2$) concentration and CD4+ count in HIV infected individuals.

levels have found to be elevated among those HIV infected individuals who have no opportunistic infection.[17] There is also evidence to show an association between high levels of virus load and increase NO production in the serum of HIV infected patients. Consequently, lymphocyte inactivation and induction of persistent immunosuppression could be a leading cause for the production of large amounts of NO by macrophages.[18]

Until the advantages of saliva-based approaches was recognized in the past decade, the value of saliva as a diagnostic tool had long been disregarded, and this led to an evolution from treating saliva as a diagnostic worthlessness to promoting salivary diagnostics. Due to rapid technology development and diagnostic capability of saliva, the gap between saliva and other bodily fluids, such as blood, urine, and cerebral spinal fluid, is closing, primarily. Salivary diagnostics would enable clinicians to monitor diseases frequently and easily and would have impact on the future medical research and therapy.[19]

In our previous study we had studied the role of nitric oxide in the pathogenesis of oral cancer, nitric oxide level was found to be increased significantly in oral cancer patients when compared with healthy subjects.[15] This shows that, elevated level of nitric oxide are involved in tumor progression.[15] Similar study was also performed to evaluate salivary nitric oxide level and dental caries where presence of NO and its metabolites in saliva of adults with natural healthy teeth was significantly higher compared to high risk group, suggesting the protective role of NO in relation to caries.[20,21] Also our study on prevalence of oral lesions in human immunodeficiency virus (HIV) positive patients showed association between number of oral lesions and CD4+ count in HIV positive patients.[1] There is a soaring need for convenient and simple point-of-care diagnostic tools in developing countries, where many health risks and illnesses remain poorly defined. Information concerning the burden of disease, guidelines for health maintenance, and disease management are seriously inadequate, resulting in severe spread of disease and high death rates. Salivary diagnostic technologies that offer easy, cost-effective, noninvasive ways to prevent disease dissemination will have enormous impact under these circumstances.[19]

Table 2 – Tukey's Multiple Comparison Test.

Groups	Mean difference	'P' value
Control vs Group I	−7.937	P < 0.001
Control vs Group II	−15.80	P < 0.001
Group I vs Group II	−7.867	P < 0.001

*P < 0.05 is statistically significant.

Our study showed that the nitric oxide ($NO_2 + NO_3$) concentration of saliva was lower in control group when compared to that of the study. Elevated levels of NO in HIV infection has been studied. Elevated levels of NO in HIV infection may be due to cytokines, mainly TNF-α and interleukin-6.

It is pertinent to note that NO is involved in HIV-1 replication, especially that induced by TNF-α. There might, therefore, be a self perpetuating mechanism wherein HIV replication leads to increased NO production and the latter increases the rate of HIV multiplication.[17]

The salivary pH and flow of stimulated and unstimulated saliva decreased significantly in early and late human immunodeficiency virus infections.[22] The total antioxidant level also decreased with decrease in CD4 count which could be used as biomarkers for correlation with disease progression in HIV infected individuals.[23] Similarly the nitric oxide ($NO_2 + NO_3$) concentration in saliva could serve as an indicator of HIV replication and progression of disease as seen in the present study.

5. Conclusion

Nearly all analytes that are in blood are also present in saliva. Although saliva has not been used as much up to this point as a sampling media, from our study it is now evident that saliva does have a strong potential to do the same kinds of tests that are done currently in blood. Salivary nitric oxide ($NO_2 + NO_3$) concentration can thus serve as an indicator of HIV replication and shows that saliva does have a strong potential to do the same kinds of tests that are done currently in blood.

Conflicts of interest

All authors have none to declare.

REFERENCES

1. Hegde MN, Hegde ND, Malhotra A. Prevalence of oral lesions in HIV infected adult population of Mangalore, Karnataka, India. *BioDiscovery*. 2012;4:1–5.
2. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991;43:109–142.
3. Nathan C. Nitric oxide a secretory product of mammalian cells. *Faseb J*. 1992;6:3051–3064.
4. Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. *Science*. 1992;258:1896–1902.
5. Blond DD, Raoul H, Grand RL. Primary human macrophages immunodeficiency virus replication in nitric oxide synthesis enhances human. *J Virol*. 2000;74:8904.
6. Evans TG, Rasmussen K, Wiebke G, Hibbs JB. Nitric oxide synthesis in patients with advanced HIV infection. *Clin Exp Immunol*. 1994;97:83–86.
7. Torre D, Pugliese A, Speranza F. Role of nitric oxide in HIV-1 infection: friend or foe? *Lancet Infect Dis*. 2002;2:273–280.
8. Chiappin S, Antonelli G, Gatti R, Elio F. De Palo a saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta*. 2007;383:30–40.
9. Slavkin HC. Toward molecularly based diagnostics for the oral cavity. *J Am Dent Assoc*. 1998;129:1138–1143.
10. Mandel ID. A contemporary view of salivary research. *Crit Rev Oral Biol Med*. 1993;4:599–604.
11. Lee Yu-Hsiang, WongSaliva DT. An emerging biofluid for early detection of diseases. *Am J Dent*. 2009;22:241–248.
12. Hooper DC, Ohnishit ST, Kean R, Numagamit Y, Dietzschold B, Koprowski H. Local nitric oxide production in viral and autoimmune diseases of the central nervous system. *Proc Natl Acad Sci*. 1995;92:5312–5316.
13. Li RH, Hotchkiss JH. Potential genotoxicity of chronically elevated nitric oxide: a review. *Mutat Res*. 1999;339:73–89.
14. Hesse M, Modolell M, La Flamme AC, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type1/type2 cytokines in vivo. *J Immunol*. 2001;167:6533–6544.
15. Hegde ND, Kumari S, Hegde MN, Shetty S. Role of nitric oxide and arginase in the pathogenesis of oral cancer. *NUJHS*. 2012;2:15–17.
16. Pacher P, Beckman JS, Liaudet L. Nitric oxide and Peroxynitrite in health and disease. *Physiol Rev*. 2007;87:315–424.
17. Wanchu A, Khullar M, Bhatnagar A, Sud A, Bambery P, Singh S. Pentoxiphylline Reduces nitric oxide production among patients with HIV infection. *Immunol Lett*. 2000;74:121–125.
18. Wanchu A, Bhatnagar A, Khullar M, Sud A, Bambery P, Singh S. Antitubercular therapy decreases nitric oxide production in HIV/TB coinfected patients. *BMC Infect Dis*. 2002;2:1–6.
19. Lee Y, Wong DT. Saliva: an emerging biofluid for early detection of disease. *Am J Dent*. 2009;22:241–248.
20. Hegde MN, Kumari S, Hegde N, Shetty S, Nireeksha. Evaluation of the status of salivary nitric oxide in patients with dental caries. *NUJHS*. 2012;2:6–9.
21. Hegde MN, Hegde ND, Ashok A, Shetty S. Salivary nitric oxide (NO_2+NO_3) as biomarker of dental caries in adults: an invivo study. *IRJP*. 2012;3:100–102.
22. Hegde MN, Malhotra A, Hegde ND. Salivary pH and buffering capacity in early and late human immunodeficiency virus infection. *Dent Res J*. 2013;10:772–776.
23. Hegde MN, Hegde ND, Malhotra A, Shetty S, Kumari S. Evaluation of total antioxidant status in HIV infected adult population of Mangalore, Karnataka, India: a biochemical study. *J Infect Dis*. 2013;112:163–167.