

## Serum Nitric Oxide Levels in Smokers with Chronic Periodontitis

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### Abstract

Smoking is a major risk factor for periodontal diseases. Nitric oxide (NO) plays a crucial role in vascular tone regulation and is involved in the pathogenesis of periodontitis. The aim of the present study was to determine and compare serum levels of NO in healthy controls, smokers and non-smokers with chronic periodontitis.

A total of 90 subjects were divided into 3 equal groups of 30 control patients, 30 non-smokers with chronic periodontitis and 30 smokers with chronic periodontitis. Periodontal disease status was determined by recording probing pocket depth (PPD) and clinical attachment level (CAL). Serum nitric oxide activity was estimated using Griess colorimetric reactions. The results were compared and statistically analyzed using ANOVA.

The mean NO levels in serum were found to be highest in smokers with periodontitis, followed by the periodontitis group, and then by the healthy controls ( $88.20 \pm 3.95$ ,  $82.29 \pm 3.54$ , and  $64.69 \pm 2.16$ ,  $\mu\text{M/L}$  respectively). The values were statistically significant between the groups ( $P < 0.001$ ).

Serum NO was significantly increased in smokers with chronic periodontitis in comparison with non-smokers. Smoking seems to amplify this effect, which could explain the severity of periodontitis in smokers. More longitudinal, prospective studies will help to verify the observations of the present study. Further research in this direction could identify reliable markers to forecast the progression of periodontitis in high-risk groups.

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### Introduction

Chronic periodontitis, characterized by the inflammation and destruction of periodontal supporting tissues, is one of the most common oral diseases.<sup>1</sup> This chronic inflammatory disease is initiated by an overgrowth of gram-negative bacteria, which results in periodontal tissue destruction and tooth loss.<sup>2</sup> The bacterial enzymes, toxins, and metabolites in the dental plaque biofilm play a key role in propagating the inflammatory process<sup>3, 4</sup>. The microorganisms associated with periodontitis are more diverse when compared with those in healthy tissue; the microbiome possesses significantly enriched

metabolic and lipid degradation pathways that are adapted for oxygen-poor environments and associated with known virulence-related activities, respectively.<sup>5,6</sup>

Smoking is a significant risk factor for the development and advancement of periodontal disease.<sup>7</sup> The negative periodontal effects and increased likelihood of established periodontal disease in smokers when compared with non-smokers have been reported previously.<sup>8,9</sup> Loss of attachment, bone, and teeth in smokers may be attributed to the destructive inflammatory effects of smoking on periodontal tissues along with the possible vasoconstrictive effects of the tobacco products; paradoxically, the signs of inflammation and bleeding during probing in smokers are mild.<sup>10-14</sup> Clinical trials have also shown that smokers respond less favorably to surgical and non-surgical periodontal treatments.<sup>15</sup>

Nitric oxide (NO) is a physiological messenger molecule involved in various physiological processes, such as the regulation

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of vascular tone, inhibition of platelet aggregation, neurotransmission, and immune response.<sup>16</sup> NO plays an important role in the progression of periodontal disease. It is a gaseous free radical that readily diffuses through the cytoplasm and plasma membrane due to its solubility in both aqueous and lipid environments.<sup>17</sup> It has a short biological half-life, and is generated enzymatically from L- Arginine by a family of NO synthase (NOS) isoforms.<sup>18</sup> NO is necessary for several biological functions such as neurotransmission,<sup>17</sup> immunoregulation, vasodilation, and cytotoxicity. In periodontal lesions, considerable quantities of NO may be generated by cells such as macrophages, PMNs, lymphocytes, and fibroblasts for prolonged durations, following induction by cytokines and lipopolysaccharides (LPS).<sup>19</sup>

Periodontitis is accompanied by an increase in the expression of inducible NOS (iNOS) and NO production in gingival tissue.<sup>20</sup> Earlier studies have reported variations in NO metabolites in patients with periodontitis.<sup>21-24</sup> To date, only one study has demonstrated an increase in serum nitrite (NO<sub>2</sub>) levels in patients with periodontitis.<sup>25</sup> However, NO<sub>2</sub> measurement alone does not represent the level of NO production because NO<sub>2</sub> is quickly oxidized to nitrate (NO<sub>3</sub>).<sup>26</sup>

Studies on NO levels in the serum of smokers with periodontitis are scarce.<sup>24</sup> Hence, this study was conducted to evaluate serum levels of NO in healthy individuals and in smokers and non-smokers with chronic periodontitis. The estimation of serum NO levels in smokers might help explain the impact of smoking on periodontitis.

### Materials and methods

A total of ninety subjects were divided into 3 groups (n = 30 each) as follows: smokers with chronic periodontitis, non-smokers with chronic periodontitis, and systemically healthy individuals (controls). The study was approved by the ethical committee of the College of Dentistry, Jazan University. All subjects were aged between 25 and 55. The periodontitis patients were selected from those referred to periodontology clinics for diagnosis and treatment, whereas individuals in the control group were selected from those who attended the pre-doctoral and staff clinics at the College of

Dentistry, Jazan University, Saudi Arabia. The periodontal status of the periodontitis patients and healthy subjects were assessed according to the classification of the American Academy of Periodontology.<sup>1, 27</sup> Smoking status was determined based on the daily consumption of tobacco.<sup>28</sup>

Patients with a periodontal probing depth  $\geq 4$  mm and clinical attachment loss  $\geq 2$  mm in at least 30% of their teeth were considered as having chronic periodontitis. Individuals who smoked a minimum of 20 cigarettes per day for no less than two years were included in the smokers with periodontitis group. Those who engaged in other forms of smoking in addition to smoking cigarettes were excluded from the study. The control group comprised individuals who had clinically healthy gingiva and no clinical attachment loss ( $\leq 3$  mm periodontal probing depth).

Informed consent was obtained from all the participants included in this study. Clinical, biochemical, and biophysical examinations were conducted to exclude any systemic illnesses. The following exclusion criteria were used: younger than 25 or older than 55 years of age; fewer than twenty-two permanent teeth; use of any type of medication (chronic or two weeks prior to the study); presence of any chronic medical condition including diabetes or infections; presence of any medical condition within the previous two weeks (flu, upper respiratory or sinus infections, allergies, skin disorders); any form of physical trauma experienced within the previous two weeks; presence of aggressive periodontitis, periodontal abscess, or necrotizing ulcerative gingivitis or periodontitis; periodontal treatment and/or antibiotic therapy received within the preceding three months; any type of dental treatment performed within the last two weeks; active carious lesions; former smokers who had quit smoking; and refusal to sign the consent form.

### Clinical Periodontal Examination

An extensive medical history was recorded based on a written questionnaire and via interviews of 20 to 30 min duration. Complete extra- and intra-oral examinations, including the number of teeth present, were documented. The third molars were excluded from the counts. All clinical measurements were performed by a single examiner. Prior to the study, calibration measurements were performed in five patients.

The intra-examiner agreement was good, with a kappa value of 0.82. Periodontal probing depth (PPD) and clinical attachment level (CAL) were measured at the mesial, distal, buccal, and lingual aspects of each tooth. Smoking history was assessed according to a standardized interview and a self-reported questionnaire. The number of cigarettes consumed per day and duration of smoking (in years) was noted.

**Venous blood samples**

Venipuncture was performed to collect 10 mL of venous blood from the antecubital fossa of each patient without excessive venous stasis. The blood samples were collected using a Vacutainer<sup>®</sup> containing no anticoagulant. The samples were then centrifuged at 3000 revolutions per minute (rpm) for 10 min. The serum samples were collected and stored in plastic vials at -70°C.

**Estimation of nitric oxide in the serum**

NO levels in the serum were estimated by measuring the levels of NO<sub>2</sub>, the stable product of decomposition, using Griess reactions as described by Green et al.<sup>29</sup> NO is a highly reactive free radical gas and is stored as NO<sub>3</sub> or NO<sub>2</sub> in the tissues. Thus, NO concentration can be estimated by measuring the concentrations of NO<sub>3</sub> and NO<sub>2</sub> in combination. The reduction of NO<sub>3</sub> to NO<sub>2</sub> by copper-coated cadmium granules followed by a Griess colorimetric reaction can be used to measure the levels of NO<sub>2</sub>.<sup>30, 31</sup>

In Griess reaction, nitrite reacts with sulfanilamide (under low pH conditions) and undergoes diazotization resulting in the formation of a diazonium salt, which then couples to N-(1-Naphthyl) ethylenediamine to form a magenta-colored azo dye with a distinctive absorption spectrum. NO<sub>2</sub> is the only stable product formed following the spontaneous autoxidation of NO in oxygenated solutions.

The Griess reagent was prepared each time before use for the estimation of NO levels using ortho-phosphoric acid (Merck KGaA, Darmstadt, Germany), purified sodium nitrite (Merck KGaA, Darmstadt, Germany), N-(1-naphthyl) ethylenediamine dihydrochloride (Merck KGaA, Darmstadt, Germany), and sulfanilamide (4-aminobenzene sulfonamide; Bio Basic Inc., Markham, ON, Canada). The serum samples were processed on the same day that the assay was performed. Each test sample (100 µL) was added to the microwells followed by the addition of 50 µL of Griess reagent 1 and 50 µL of Griess

reagent 2; the agents were mixed thoroughly and incubated for 10 min at room temperature. Subsequently, the absorbance was read at 540 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The nitrite content in the samples was determined using a standard curve constructed from known standard concentrations. A linear regression equation was employed to ascertain the corresponding absorbance values.

**STATISTICAL ANALYSIS**

Statistical analysis of the data was performed using GraphPad<sup>®</sup> (GraphPad Software, Inc., San Diego, CA). Means and standard deviations for age, periodontal probing depths, clinical attachment levels, and serum NO levels were analyzed, and differences among them were determined using one-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparisons post hoc test. P-values <0.05 were considered significant. Student's t-test was used to analyze the mean differences in periodontal probing depth and clinical attachment levels between the two periodontitis groups.

**Results**

Table 1 illustrates the distribution of the study population based on age, CAL, and PPD. The mean PPD and CAL values measured at six sites were calculated for each individual in the smoker and non-smoker with periodontitis groups (Table 1). The smoker group had slightly higher PPD and CAL values than the non-smoker periodontitis group, statistical significance notwithstanding.

Groups (n=30)	Age (years)	CAL (mm)	PPD (mm)
	Mean ± SD	Mean ± SD	Mean ± SD
Healthy	34.25 ± 7.95	--	0.93±0.39
Periodontitis	34.45 ± 7.42	3.38 ± 0.44	5.54 ± 0.34
Smokers with Periodontitis	35.75 ± 6.74	3.73 ± 1.03	5.57 ± 1.03

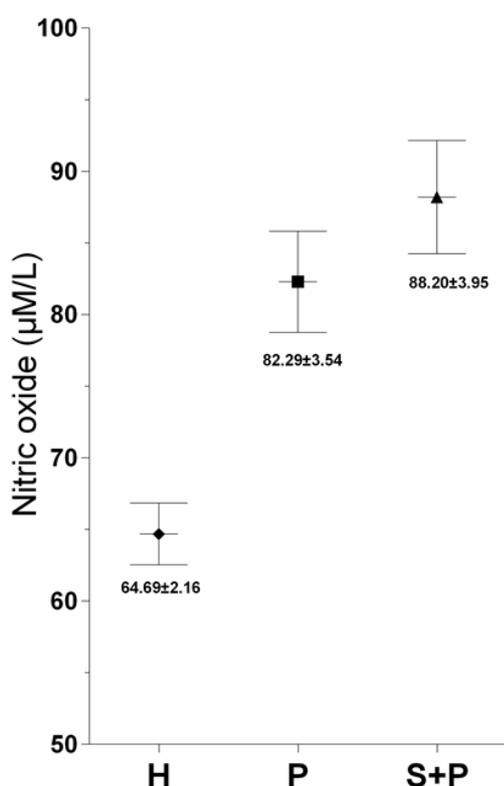
**Table 1.** Study population data including age, clinical attachment level (CAL), and probing pocket depth (PPD).

Serum NO levels in the subjects are shown in Table 2 and Figure 1. Mean serum NO concentration was found to be significantly higher in the smokers with periodontitis (88.20±3.95) when compared with the non-smokers with

periodontitis ( $82.29 \pm 3.54$ ;  $P < 0.05$ ). Both groups had relatively higher serum NO levels than the healthy controls ( $P < 0.001$ ).

Groups	Nitric oxide ( $\mu\text{M/L}$ ) (Mean $\pm$ SD)
Healthy	$64.69 \pm 2.16$
Periodontitis	$82.29 \pm 3.54$
Smokers with Periodontitis	$88.20 \pm 3.95$

**Table 2.** Serum levels of nitric oxide in smokers and non-smokers with periodontitis.



**Figure 1.** Serum nitric oxide levels in smokers and non-smokers with periodontitis. (H-Health Controls, P- Periodontitis, S+P Smokers with periodontitis).

### Discussion

Smoking is one of the strongest risk factors for periodontal disease. The toxic components of tobacco smoke, particularly nicotine, can directly or indirectly harm the tissues of the periodontium<sup>14</sup>. Smokers are more likely to harbor higher levels of potential periodontal pathogens. Moreover, smoking is

known to impair various aspects of both innate and acquired immune responses.<sup>32, 33</sup>

NO is a free radical synthesized by activated inflammatory cells. It regulates the function of other cells involved in the inflammatory process and appears to act as secondary mediator of some actions of certain pro-inflammatory cytokines, such as IL-1. The prolonged production of pro-inflammatory mediators may result in increased NO production and the stimulation of cyclooxygenase (COX-II), resulting in PGE<sub>2</sub> synthesis and tissue catabolism. The inflammatory response is largely self-limiting; however, it can be unrelenting leading to excessive tissue damage when dysregulated or when the causative agent persists, as in periodontal disease conditions.<sup>19</sup>

Nicotine has been shown to activate PMNs, suggesting a systemic inflammatory reaction. The periodontal tissues are infiltrated mainly by neutrophilic granulocytes and PMNs, which play an important role in the development of inflammatory injury. Tobacco-induced degranulation events in neutrophils, tobacco-induced alterations to the microbial flora, increases the proinflammatory cytokine burden in smokers contributing to the development of inflammatory damage.<sup>34</sup> Studies have shown that oxidative stress and changes in NO formation or action play a major role in chronic inflammatory diseases.<sup>35</sup> In the present study, we indirectly measured serum NO levels by estimating the NO<sub>3</sub> levels and stable end products of NO oxidation using Griess reaction. It has been reported that endogenous NO production is highly correlated with NO<sub>3</sub> and NO<sub>2</sub> levels in serum and plasma. Hence, the estimation of NO<sub>3</sub> and NO<sub>2</sub> is a relative measure of NO production *in vivo*.<sup>30, 36</sup>

Elevated NO production in chronic periodontitis is a reflection of an immune-activated state during which inflammatory cytokines and other mediators are up regulated. Cytokine- and bacterial toxin-induced NO up regulates IL-1, TNF- $\alpha$ , and IL-8 production by neutrophils, and this is a potential mechanism for the escalation of inflammation in periodontal disease.<sup>23</sup> NO has been shown to directly activate both constitutive and inducible forms of the cyclooxygenase (COX) enzyme, leading to an increase in PGE<sub>2</sub> production and enhanced bone resorption in periodontal disease.<sup>37, 38</sup>

There is a lack of consensus regarding the precise mechanism for the pathogenesis and progression of periodontitis in smokers. Smoking has a negative impact on various aspects of the innate and adaptive immune responses, resulting in altered neutrophil function, fibroblast activity, antibody production, vascular factor production, and inflammatory mediator production.<sup>33,39-45</sup> Therefore, it is probable that these effects on the immune system could be the primary factors associated with smoking that contribute to the pathogenesis of periodontal disease.<sup>39</sup>

The results of the present study showed higher levels of NO in serum from smokers when compared with the non-smokers with periodontitis. Although earlier studies have shown higher NO levels in subjects with periodontitis,<sup>25,37</sup> only one study has examined NO levels in smokers with severe periodontitis.<sup>24</sup> NO production increases during the deposition of plaques, which might be an early host defense mechanism against bacterial proliferation.<sup>46</sup> NO is known to potentiate matrix degradation, including the suppression of proteoglycan and collagen synthesis and up regulation of metalloproteinases activity.<sup>19,47,48</sup> Based on an examination of the available data, NO appears to play an important role in the immuno-inflammatory process. Hence, modulation of this mediator has a potential role in the treatment of periodontitis. The selective inhibition of NO synthesis and either the scavenging of peroxynitrite or the neutralization of reactive species by anti-oxidant substances appear to be novel and promising approaches for the treatment of periodontitis.<sup>49,50</sup>

## Conclusions

The results of this study demonstrated higher serum NO levels in smokers with chronic periodontitis when compared to the non-smokers with the periodontitis and the healthy individuals in the control group. Despite the limitations of the study, it can be concluded that higher levels of NO in the serum could be a cause or effect of the severity of periodontitis in smokers. Further studies may help in the identification of reliable markers that can forecast the progression of periodontitis in high-risk groups.

## Abbreviations

NO: Nitric oxide; PPD: Probing pocket depth; CAL: clinical attachment level; PMN:

polymorphonuclear leukocytes; LPS: Lipopolysaccharide; NO<sub>2</sub>: nitrite; NO<sub>3</sub>: nitrates; COX: cyclooxygenase; PGE<sub>2</sub>: Prostaglandin E<sub>2</sub>.

## Declaration of Interest

The authors report no conflict of interest.

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