Would Nitric Oxide be an Effective Marker for Earlier Stages of Peri-Implant Disease? An Analysis in Human Peri-Implant Sulcular Fluid

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Nitric oxide has an important effect on host immune response. However, little has been studied in relation to its potential as a possible diagnostic tool in peri-implant disease. The present study analyzed nitrite levels in the peri-implant sulcular fluid (PISF) of implants with mucositis and the correlation of these nitrite levels with clinical parameters using a simplified fluid collection methodology. Twenty-five partially edentulous patients showing peri-implant mucositis were evaluated, and the peri-implant status was determined based on current clinical parameters: probing depth (PD) and bleeding on probing (BOP). The sulcular fluid (SF) around teeth (control) and implants were collected, and the nitrite levels were evaluated using the Griess method. The mean probing depth (mm) was significantly higher ($P < .0001$) in implants ($2.852 \pm 0.6484$) than in control teeth ($1.585 \pm 0.3636$). The mean total nitrite level ($\mu$M) was statistically higher ($P = .0069$) in implants with mucositis ($14.34 \pm 11.83$) than in control teeth ($9.316 \pm 5.534$). No correlation was observed between the total nitrite levels and the PD mean in the control group ($P = .2558, r = -0.2361$) or in the implant group ($P = .1160, r = -0.3224$), as well as the number of faces showing bleeding on probing ($P = .8747, r = 0.0332$). These results demonstrated that the nitrite levels were higher in inflamed areas. According to the methodology applied and results obtained, the higher nitrite levels in inflamed areas suggest that, in the future, nitrite could be used as a marker of peri-implant mucositis associated with clinical data to monitor the cure or evolution of the disease.

**Key Words:** dental implant, diagnosis, peri-implant sulcular fluid, peri-implant mucositis, nitrite

**INTRODUCTION**

In modern dentistry, endosseous oral implants have become an important therapeutic modality for replacing missing teeth. The success rate is high, and long-term studies have shown low rates of oral implant failures.\textsuperscript{1-4} Despite this high success rate, early failures may occur as a result of lack of osseointegration due to interference with the healing process. Late failures may occur when the achieved osseointegration is lost after a period of function due to peri-implantitis, occlusal overload, or implant fracture. Thus, optimal oral hygiene and proper occlusion are considered critical to the long-term success of endosseous oral implants.

The monitoring of dental implants is crucial for the prevention or early detection of inflammation since it progresses more rapidly in implant sites than in teeth.\textsuperscript{5-7} In general, peri-implant health is evaluated using a variety of clinical tools and image-based methods.\textsuperscript{7} The frequent clinical measures applied include radiographs, bleeding on probing,
probing depth, and mobility. These clinical parameters do not reveal current disease status. Therefore, the development of reliable methods is essential for the early detection of change in peri-implant status prior to irreversible bone loss.

Diagnostic tools based on biological markers from sulcular fluid and saliva have been extensively investigated in periodontal disease. The similarities between the periodontal and peri-implant disease site led the researchers to investigate host-derived diagnostic markers of periodontitis, identified in sulcular fluid, that are the same in the peri-implant sulcular fluid.

Nitric oxide (NO) is a molecule with multiple functions, involved in both physiologic and pathologic conditions of the periodontal and peri-implant region. Nitric oxide also has regulatory functions in inflammation, including modulation of vascular tone, microvascular permeability, and leukocyte migration. In periodontal tissues and saliva, it may be a part of the nonspecific natural defense mechanisms against pathogenic bacteria. Nevertheless, NO toxicity is not restricted to microbes, and high production of NO can lead to tissue destruction at periodontal and peri-implant sites.

Since NO is an unstable molecule that is short-lived in biologic systems due to its spontaneous autoxidation, it can be measured using nitrite. This end metabolite of nitric oxide can be quantified by direct means, reflecting the production, bioavailability, and metabolism of NO in vivo. Quantification of nitrite levels associated with clinical parameters might be useful in the early diagnosis of tissue destruction and in the prognosis of implants. A potential diagnostic tool would provide a routine assessment method at dental offices. The present study analyzed nitrite levels in the peri-implant sulcular fluid (PISF) of implants with mucositis and the correlation of nitrite levels with clinical parameters using a simplified fluid collection methodology.

**Materials and Methods**

**Patient’s selection**

The present study was planned as an internal cross-sectional study to comparatively analyze dental implants with apparent clinical inflammation (mucositis) and healthy teeth (control) in the same individual. Twenty-five partially edentulous individuals (12 men and 13 women) aged 25–45 years and who had dental implants placed over the last 10 years were selected from a private clinic. All implants had been installed by the same surgeon and had been in function for at least 1 year. The criteria for the selection of subjects were the absence of systemic diseases, no antibiotic treatment for at least 3 months before sampling, no chronic corticosteroid use, non-smokers, no history of periodontal or peri-implant therapy for at least 3 months, and the presence of at least one endosseous dental implant restored with an appropriate prosthesis. To minimize interpatient variation, 1 implant (restored and in function) and 1 healthy tooth (intrinsic control) were selected in the same individual. The protocol for examining the patients and collecting fluid samples was approved by the ethical committee of the Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil (370/06), and all patients gave informed consent to participate in this study.

**Determination of the clinical status of the soft tissue**

The clinical status of the dental implant and control teeth was evaluated by assessing the probing depth (PD) and bleeding on probing (BOP). Clinical measurements were taken at 6 sites: distobuccal (DB), center-buccal (CB), mesiobuccal (MB), distolingual (DL), center-lingual (CL), and mesiolingual (ML). PDs were recorded from each site using a conventional UNC periodontal probe (Hu-Friedy, Chicago, Ill). Care was taken to avoid any physical injuries in the sulcular area. Radiographic examinations were obtained to confirm the absence of apical lesions and bone loss around the teeth and implants. The clinical examinations were performed by a single examiner.

**Determination of the experimental group—peri-implant mucositis**

In each subject, the implants were selected based on PD and BOP. Implants that showed BOP and PD >3 mm in at least one site and an absence of bone loss confirmed by radiography were classified as having mucositis. The scores utilized for BOP were 0 for no bleeding when the periodontal probe was passed along the gingival margin and 1 for bleeding on the gingival margin. The probing depth values
were categorized as: I, $\leq 3$ mm; II, $>3$ mm and $<5$ mm; III, $\geq 5$ mm. In addition, a healthy tooth (intrinsic control) showing no clinical evidence of periodontal disease (BOP $= 0$ and PD $\leq 3$ mm) was selected.

**Sulcular fluid**

The same protocol was used to collect the sulcular fluid (SF) samples from teeth and implants as follows. After removing supragingival plaque, the sites were isolated with sterile cotton rolls and dried with a gentle stream of air to reduce any contamination with plaque or saliva. The fluid samples were collected from the 6 sites (DB, CB, MB, DL, CL, and ML) of each implant and tooth using standardized endodontic paper.\textsuperscript{29} size 30 (Maillefer, Dentsply, Switzerland). The absorbent paper was inserted gently into the gingival sulcus until slight resistance was felt. Each paper was left in place for 30 seconds. Then, all endodontic papers from the same patient (tooth or implant) were placed into 1.5-mL plastic tubes containing 300 $\mu$L of elution buffer (50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 1 mM CaCl\textsubscript{2}). To extract the sulcular fluid collected on the endodontic paper, each sample tube was centrifuged 2 times at 10 000g for 300 seconds, the absorbent papers were discarded, and the supernatants were stored at $-20^\circ$C prior to analysis. Therefore, for each patient, 2 sample tubes containing 6 endodontic papers were obtained, including 1 wet paper per site. One tube contained the SF of the tooth and the other, peri-implant fluid.

**Determination of NO$_2^-$ based on the Griess method**

The production of NO was determined by measuring the sulcular fluid's total nitrite (NO$_2^-$) using the Griess method.\textsuperscript{30} Equal amounts of 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid were combined to create the Griess reagent. In an optically clear 96-well plate, 100 $\mu$L of the Griess reagent was added to 100 $\mu$L of the sample. The plates were incubated at room temperature to allow the development and stabilization of chromophore. Absorbance was measured at 540 nm with a microplate reader. Linear regression analysis was used to calculate the nitrite concentration in the sulcular fluid samples from the standard calibration curves of sodium nitrite (NaNO$_2$; 200–0.1 $\mu$M). The sample nitrite levels were expressed as NO$_2^-$ ($\mu$M) and statistically analyzed. As a reaction control, endodontic papers submerged in buffer were used.

**Statistical analysis**

GraphPad Prism was used for all statistical analyses. Normality of distribution was tested for clinical parameters, and nitrite levels were assessed at the implants and at the control teeth. Since the data were not normally distributed, comparisons between healthy teeth and mucositis implants were made using the Mann-Whitney U test with Bonferroni correction. The comparisons between teeth and dental implants were evaluated as the average value of the sampled sites per implant or tooth. The correlations between nitrite levels and recorded clinical parameters were analyzed using Spearman correlation test. $P < .05$ was considered statistically significant.

**Results**

The implants with mucositis and the control teeth were classified according to the clinical parameters (PD and BOP). For categorical variables, the results are presented as numbers, while for continuous variables, the mean (\(\mu\)) and standard deviation are presented. The mean probing depth (mm) was significantly higher ($P < .0001$) (Figure 1a) in implants with mucositis (2.852 $\pm$ 0.65) than in the matching control teeth (1.59 $\pm$ 0.36). All evaluated implants showed BOP of at least 1 site. The mean total nitrite level ($\mu$M) was higher in implants with mucositis (14.34 $\pm$ 11.83) than in control teeth (9.316 $\pm$ 5.534), showing a statistically significant difference ($P = .0069$) (Figure 1b). No correlation was observed between the mean total nitrite levels and the PD mean in the control group ($P = .2558$, $r = -0.2361$) (Figure 2a) or in the implant group ($P = .1160$, $r = -0.3224$) (Figure 2b), as well as the number of faces showing bleeding on probing ($P = .8747$, $r = 0.0332$). A preliminary test correlating the total nitrite levels and the different categories of PD of the implants (number of sites with PD $\leq$ 3 mm, $>$ 3 mm, or $\geq$ 5 mm), confirmed the lack of correlation between NO and PD ($P < .0001$).
Despite the high rates of dental implantation, there is concern regarding the maintenance of a healthy peri-implant condition since late failures are caused mainly by peri-implant disease. In general, clinical exams as well as radiographic signs of bone loss are used for the diagnosis and monitoring of peri-implant disease progression. However, these methods provide limited information about the dynamic pathophysiologic mechanisms of disease initiation and progression. Therefore, simple and objective tests based on biologic markers may be important to monitor peri-implant tissue health during recall visits.

The present study used traditional clinical parameters to determine the peri-implant status in patients with different dental implant systems. These parameters were associated with the quantification of the biochemical marker of inflammation and bone-remodeling nitric oxide, which was quantified in the sulcular fluid of teeth and implants as the total nitrite level. Previous studies have already evaluated nitric oxide levels around dental implants, demonstrating the role of this molecule in the inflammatory process of peri-implant soft tissues and bone turnover.

In the present research, the samples were collected using absorbent endodontic papers, which are accessible to all clinicians, and all fluid samples were grouped in the same device, which reduced the number of procedure steps. This simplification of the fluid collection methodology seems closer to the routine of dental offices. A preliminary test was realized using only endodontic papers submerged in buffer.
to ensure that elements of the endodontic paper did not have any influence on nitrite levels. The methodology used in this study also made the use of Periotron unnecessary. This equipment is not commonly found in dental offices, despite its importance in studies that require fluid volumetric determination. The data were presented as total nitrite levels, which were considered by Yamalik et al34 and Tözüm et al18,19 as an appropriate method of presentation.

The clinical parameters (PD and BOP) are simple and fast methods often used during follow-up visits, taking into account the real conditions of private dental offices. Despite the limitations of these 2 parameters, they have often been reported in previous studies to determine peri-implant status.6,10 The implants generally have a greater probing depth than the teeth,35,36 due to the anatomic and histologic differences between teeth and implants.4,8,36,37 Around implants, the gingival tissues form a tight adhesion, similar to the junctional epithelium around the teeth.4 However, the lack of cementum as well as the absence of perpendicular and circular collagen fibers in peri-implant connective tissue allows the periodontal probe to pass easily apically to the epithelial junction.4,8 As result, the PD measurements are higher in implants than in natural teeth, especially when inflammation is present.4,8,36,37 Since the proteolytic enzymes present at inflamed sites cause the destruction of collagen fibers. BOP may not be an accurate indicator of peri-implant inflammation since this parameter may occur more incidentally, being even more severe around healthy dental implants than in periodontal tissues.4,37 Another factor to consider is that calibrated probes were not used in this study. Although calibrated probes allow the same force to be used for all implants and teeth, most offices do not have such equipment. Since a method of diagnosis and prevention on a large scale should be easily accessible to professionals in developing countries, we chose to simplify the methodology using conventional probes and a precalibrated examiner.

In the present study, the inflamed implants showed higher nitrite levels, as reported by Tözüm et al.18,19 These data confirm what is already well-documented regarding the role of nitric oxide in the pathogenesis of inflammation.38 The 2 main pathways of production of oral NO and nitrite are chemical from the physiologic reduction of dietary nitrate and enzymatic from the conversion of L-arginine by inducible nitric oxide synthase (iNOS), one of the isoforms of NOS.22 Furthermore, much of the nitrite of body fluids is formed by the oxidation of NO produced by iNOS.18 This enzyme is expressed by nerves, salivary glands, and endothelial and inflammatory cells and may be induced by proinflammatory stimuli, resulting in the production of high amounts of nitric oxide for a long period. In general, iNOS expression in healthy adult tissues is absent.20 However, even clinically healthy gingival tissues present an inflammatory infiltrate; thus, the control teeth also showed detectable amounts of nitrite levels but in quantities lower than when compared with the levels of nitrite in the implants with mucositis. Previous studies have demonstrated increased iNOS expression in gingivitis and in chronic and aggressive periodontitis.

Total nitrite levels did not show a correlation with the clinical parameters evaluated, which was also observed by Tözüm et al.18,19 in the data presented both per implant and per site. This lack of significant correlations between the laboratory findings and clinical status suggests a temporal difference between clinical and biologic changes.18,19 Therefore, the chair-side simple methodology proposed in the present study may be useful in the future to collect fluid samples and quantify nitrite levels, using a healthy tooth from the same patient as a control. Although previous studies have compared the levels of nitrite in healthy and inflamed implants, we chose to use teeth as a control. The choice of a healthy tooth for comparison was based on the fact that it is necessary to create a diagnostic tool that applies both to individuals with multiple implants and those with one implant. Although not used in this study, it would also be interesting to use an extrinsic control consisting of fluid from healthy teeth collected from individuals without caries and periodontal inflammation. This type of control would follow the detection system and be used as a control for laboratory analysis.

The importance of these findings may be that, in the absence of traditional parameters such as BOP or gingival erythema, NO may indicate a risk or lack of risk for disease progression at implants. It appears that the NO test may be useful as an adjunct to traditional clinical parameters. The results
of this investigation should be interpreted with caution due to the cross-sectional design. We suggest that longitudinal monitoring of NO in PISF may confirm its possible use as a marker and/or predictor of implant failure.

**CONCLUSION**

With the methodology applied, the results showing higher nitrite levels in inflamed areas suggest that in the future nitrite could be used as a marker of peri-implant mucositis associated with clinical data to monitor the healing or evolution of the disease.

**ABBREVIATIONS**

BOP: bleeding on probing
CB: center-buccal
CL: center-lingual
DB: distobuccal
DL: distolingual
iNOS: inducible nitric oxide synthase
MB: mesiobuccal
ML: mesiolingual
NO: nitric oxide
PD: probing depth
PISF: peri-implant sulcular fluid
SF: sulcular fluid

**REFERENCES**


28. Tözüm TF, Turkyilmaz I, Yamalik N, Karabulut E, Eratalay K. Analysis of the potential association of implant stability, laboratory,


