# Analysis of the Possible Impact of Inflammation Severity and Early and Delayed Loading on Nitric Oxide Metabolism Around Dental Implants

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***Purpose:*** *The aim of the present study was to analyze the possible impact of clinical status, presence and severity of inflammation, and loading on nitric oxide (NO) metabolism around mandibular dental implants.* ***Materials and Methods:*** *A total of 34 implants in 17 patients, loaded either early (EL) or after a delay (DL), were classified according to the presence and severity of clinical inflammation in the peri-implant sites. Clinical parameters were recorded, peri-implant sulcular fluid (PISF) samples were obtained, and PISF nitrite levels were spectrophotometrically determined. Clinical measurements and nitrite analysis were repeated at 1, 3, 6, and 9 months postloading at available sites.* ***Results:*** *Compared to noninflamed sites, inflamed sites demonstrated higher mean total nitrite levels (*P = *.032) that tended to increase with the severity of inflammation at both EL and DL implants. At noninflamed sites, EL implants provided significantly higher PISF volume than DL implants (*P = *.001). At noninflamed sites, EL implants revealed higher total nitrite levels; on the contrary, at inflamed sites, DL implants revealed higher total nitrite levels. In general, nitrite levels demonstrated a pattern of decrease followed by an increase during follow-up.* ***Discussion:*** *Increased NO production with the presence and the severity of inflammation supports the contribution of NO in the peri-implant inflammatory process. Loading is also likely to have an impact on NO metabolism, which suggests a role for NO in remodeling and adaptation of bone around dental implants.* ***Conclusion:*** *Besides the presence of inflammation, the severity of inflammation and loading also seem to have an impact on NO metabolism around dental implants.* (50 references) Int J Oral Maxillofac Implants 2005;20:547–556

**Key words:** *dental implants, implant loading, inflammation, nitric oxide, nitrite*

Reactive oxidants generated by phagocytic white blood cells play critical roles in host defense by destroying invading pathogens and tumor cells. Nitric oxide (NO) is such a diatomic free radical produced in copious amounts by activated phagocytic leukocytes. Two major forms of NO synthases, constitutive and inducible isoforms (cNOS and iNOS), catalyze the synthesis of NO from arginine. cNOS isoforms are involved in short-lasting and low-level synthesis of NO; however, iNOS (the isoforms found in phagocytic cells) is responsible for long-lasting and high-level synthesis. NO entering red blood cells rapidly reacts with oxyhemoglobin to form nitrate. Another end metabolite of NO is nitrite, which is formed from spontaneous and rapid autoxidation reactions of NO in aerobic solutions. Since autoxidation of NO is dependent on its own concentration, nitrite generation predominates at the site of inflammation.[1]

It is well recognized that NO plays a vital role in many biologic systems and is essential for a vast spectrum of intracellular and extracellular events. These include homeostatic functions, including vasodilatation; inhibition of platelet adhesion and

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aggregation; neurotransmission; and host defense against infections agents such as parasites and fungi.[2–4] It has the ability to pass freely within and between cells; thus, it is involved in cell-to-cell communication.[5]

Although NO is important in host defense and homeostasis, it is also regarded as harmful and has been implicated in the pathogenesis of inflammation.[2–4,6] Briefly, NO exerts damaging effects via direct cytotoxic or cytostatic actions such as the stimulation of the release of proinflammatory mediators such as peroxynitrite, which mediates the cytotoxic effects of NO or interleukin-6, or tumor necrosis factor and interferon-γ, which are capable of stimulating NO production in bone cells.[6–8] NO also has a significant role in the inflammatory response in arthritic joints in humans, and it is clear that it is involved in both acute and chronic inflammation.[9,10]

Nitrate is rapidly reduced to nitrite in the oral cavity by microbial nitrate reductase enzyme activity, especially the activity of enzymes on the surface of the tongue and around teeth and periodontal tissues.[11–13] In the human oral cavity, oral neutrophils produce larger amounts of NO compared to peripheral neutrophils.[14] Nitrite in saliva may have an effect on the growth and survival of the bacteria implicated in periodontal disease, including *Fusobacterium nucleatum, Eikenella corrodens,* and *Porphyromonas gingivalis*.[11] Likewise, in an experiment in which ligatures were used to induce periodontitis in rats, iNOS was expressed at higher concentrations at sites where periodontitis had been induced in comparison with nonligated control periodontal sites.[15]

A greater level of iNOS expression was demonstrated in both gingival inflammation and periodontally diseased tissues than in healthy controls.[16,17] In both gingival inflammation and periodontally diseased tissues, it has been suggested that macrophages and endothelial cells contribute to NO production.[16,17] The expression of high levels of iNOS from macrophages was also reported to damage the periodontal tissues in patients with localized aggressive periodontitis (LAP),[7] and iNOS activity in macrophages was suggested to have the potential to inhibit leukocyte recruitment by acting on leukocytes themselves or on endothelial cells that may attenuate detrimental inflammatory response in LAP.[7] Further, polymorphonuclear cells presented an additional activation pathway in periodontal disease, expressing significant iNOS and representing an important source of NO in human plaque-induced gingivitis and localized chronic periodontitis.[18] Moreover, NOS was present in human neutrophils and NOS activity in LAP neutrophils was increased where it was negatively correlated to chemotaxis response.[19]

Where bone remodeling in response to mechanical strain or stress is concerned, in vitro and in vivo studies have provided evidence that fluid flow–induced shear stress rapidly stimulates NO release from osteoblasts, preosteoclast-like cells, and osteocytes.[20,21] Thus, it has been speculated that fluid flow-induced shear stress NO production could play a primary role in bone maintenance and remodeling.[20] Where hip replacement and aseptic loosening of orthopedic implants are concerned, there is a trend toward a moderate increase in immunohistochemically iNOS-positive cells in tissues containing particulate wear implant debris, and a statistical correlation between iNOS and the severity of osteolysis around prosthetic hip implants has been determined.[6] Orthodontic models also suggest the role of NO in the bone remodeling cycle.[22,23] When closed-coil spring orthodontic appliances were applied and L-arginine (a NO precursor) was injected, a significant increase in tooth movements and an increased number of osteoclasts were observed, while L-NAME (NOS inhibitor) injection resulted in a significant reduction in orthodontic tooth movements.[22] Furthermore, after rat molar tooth movement, increased amounts of iNOS were produced in the periodontal ligament and connective tissue between roots of moved teeth as well as around blood vessels, where it has been speculated that NO may have a role in periodontal remodeling during tooth movement.[23] All available data suggest that NO is an important biochemical mediator in the response of periodontal tissue to orthodontic forces and that it has a primary role in the bone remodeling cycle.[22,23]

Although the impact of NO production on the bone remodeling cycle and the physical and pathologic features of NO production in healthy and diseased periodontal sites have been demonstrated, and research has been conducted on orthopedic implants in the medical arena, quantitative analysis of NO production in regard to dental implants in human subjects had apparently not yet been performed. Therefore, through the quantifying of the peri-implant sulcular fluid (PISF) nitrite levels, a stable end product of NO oxidation, the present study aimed to determine the possible effect of clinical inflammation and also the loading of dental implants on NO metabolism around dental implants.

## MATERIALS AND METHODS

### Patients and Presurgical Evaluation

Seventeen edentulous patients, 9 women and 8 men, referred to Department of Prosthodontics, Faculty of Dentistry, Hacettepe University, seeking prosthodon-

tic rehabilitation were included. All patients received the treatment protocol and signed an informed consent form. Their medical history was unremarkable; they had no known allergies and no known metabolic bone diseases, and their ages varied between 42 and 65 years, with a mean of 53 years. They were provided with detailed information regarding the procedure. After consent had been obtained, they were also introduced to the surgical procedure and the application of a complete mandibular prosthesis with 2 ball attachments. Prior to surgical procedure, dental computerized tomography (CT) was used to determine the ideal implantation sites in the mandibular anterior region of all subjects. All patients then received 2 mandibular endosseous dental implants 15 mm in length and 3.75 mm in diameter (Brånemark System, Nobel Biocare, Göteborg, Sweden). The same oral surgeon performed all implant placement surgeries.

### Surgical Procedure and Prosthodontic Rehabilitation

Ultracain D-S (Hoechst Marion Roussel, Frankfurt/Main, Germany) was administrated bilaterally to the anterior mandibular area. Initially, full-thickness flaps were reflected and implant osteotomies were located 5 mm anterior to the mental foramina as determined on dental CT prior to surgical procedure. Following the placement of implants in their prepared sites, resonance frequency values of both implants were measured by Osstell (Integration Diagnostics, Göteborg, Sweden). The transducer was mounted on the implants orthoradially, with the upright part on the oral side. It was tightened with a screw by hand. The frequency response of the system was measured immediately after implant placement to determine the stability of implants.[24] Implant stability quotient values more than 65, which indicated high primary stability, were included in the study to provide a standardized methodology.[25] The flaps were then closed with 4-0 sling sutures. Following surgery, patients were given a cold compress extraorally to minimize swelling and bleeding. A week after surgery, uneventful healing was observed in all cases. A total of 34 mandibular dental implants were randomly divided into 2 groups based on whether they were loaded early (EL; n = 18) or after a delay (DL; n = 16). In the EL group, abutment connection was performed immediately after surgery and a definitive mandibular prosthesis was introduced within 5 days; in the DL group, abutment connection and prosthesis delivery occurred 3 months after surgery. The same prosthodontist provided all prostheses.

### Clinical Evaluation

Clinical status of the dental implant sites was evaluated by assessing the probing depth (PD),[26] Plaque Index (PI) score,[27] Gingival Index (GI) score,[28] and the gingival bleeding time index (GBTI).[29] All measurements were performed at 4 sites around each implant (mesial, distal, buccal, and lingual) and were carried out to the nearest mm using a Michigan "O" probe. All measurements were performed after PISF sampling. Care was taken not to cause any physical irritation inside sulcular area.[30] Clinical evaluations were performed by the same periodontist.

### Determination of Experimental Groups

To analyze the potential effect of presence or absence of clinical inflammation on PISF nitrite levels, dental implant sites were classified as either clinically healthy sites or sites with inflammation based on the GI score of a given implant site. A GI score of 0 was considered to represent the state of clinical health (no clinical inflammation); a GI score of $> 0$ represented clinical inflammation. In neither clinically healthy nor inflamed sites did radiographic examination reveal detectable bone loss around the dental implants. Dental implants were further divided into 3 subgroups according to the severity of the clinical inflammatory status (based on the recorded GI scores): healthy (GI = 0), slightly inflamed ($GI \leqslant 1$) and moderately/severely inflamed ($GI > 1$).

### PISF Sampling

At 1, 3, 6, and 9 months postimplantation, PISF sampling was performed at the 2 mandibular dental implant sites. PISF samples were obtained according to the method described by Rüdin and associates[31] using standardized paper strips (Periopaper, no. 593525; Ora Flow, Amityville, NY). In brief, the sampling area was isolated with cotton rolls, supragingival plaque was removed, and the site was gently air-dried. To reduce the risk of mechanical irritation and to avoid changing the actual PISF volume in a given site, the placement of paper strips and the duration of PISF sampling were standardized. At each site, standardized Periopaper strips were inserted 1 mm into the peri-implant sulcus (regardless of the probing depth of the area) and left for 30 seconds. This was done to minimize the risk of mechanical irritation, to eliminate the risk of bleeding, and to further standardize the PISF sampling procedure. Samples with evidence of gingival bleeding were excluded. To eliminate the risk of evaporation, paper strips with PISF were immediately transported to a previously calibrated Periotron 8000 (Ora Flow) for volume determination.

Prior to sampling, the Periotron 8000 was switched on and allowed to warm up before a blank Periopaper strip was placed in the device and the reading dial was set to zero.[32] Calibration of the Periotron 8000 was checked periodically and by the performance of triplicate readings as described by Deinzer and colleagues.[33] Following sampling, the PISF collected was measured in Periotron units, which were converted to microliters by MLCONVERT.EXE software (Ora Flow).[33] The PISF samples were then placed in sterile, firmly wrapped Eppendorf tubes and stored at –20°C until the day of laboratory analysis.

### Determination of Nitrite Level of Sulcular Fluid

To each PISF sample in the Eppendorf tube, 130 µL distilled water was added, and the samples were vigorously mixed for the extraction of nitrite into water. Then 100 µL of the extract was mixed with 0.5 mL of freshly prepared Griess reagent. After 10 minutes' incubation at room temperature, the absorbance of each sample was determined at 540 nm.[34] A standard curve was prepared using sodium nitrite to calculate the nitrite concentration in PISF.

### Statistical Analysis

SPSS 11.5.0 software for Windows (SPSS, Chicago, IL) was used for all statistical analysis. For clinical parameters and PISF nitrite levels in healthy and inflamed peri-implant sites, the Shapiro-Wilk test was used to test the normality of distribution.[35] Since data were not normally distributed, the Mann-Whitney test was performed for comparison of healthy and inflamed peri-implant sites,[36] while the among-group comparisons (healthy, slightly inflamed, and moderately/severely inflamed sites) were analyzed using the Kruskal-Wallis test. For pairwise comparisons, the Mann-Whitney test with Bonferroni correction ($\alpha$/number of pair-wise comparisons) was used.[37,38] The correlation between nitrite levels and clinical parameters was analyzed with Spearman's correlation coefficient.[39]

For clinical parameters and PISF nitrite levels according to the implant loading protocol, the Shapiro-Wilk test was used to test the normality of distribution.[35] Since data were not normally distributed, Kruskal-Wallis analysis followed by the Mann-Whitney test with Bonferroni correction ($\alpha$/number of pair-wise comparisons was performed for the comparison of healthy and inflamed sites for EL and/or DL implants.[36–38] The correlations between nitrite levels and recorded clinical parameters were analyzed with Spearman's correlation coefficient.[39]

Although the basic study design was cross-sectional, clinical parameters and PISF sampling were repeated for the available implant sites at 1, 3, 6, and 9 months following surgery. However, because of the ongoing nature of the longitudinal part of the present study and the limited number of available implant sites during follow-up visits, no statistical analysis could be performed, and only the preliminary results are reported. *P* values less than .05 were considered statistically significant.

## RESULTS

### Analysis of Implants Grouped by State of Inflammation

Table 1 shows the data for the recorded clinical parameters, PISF volume, total nitrite levels, and nitrite concentration. All clinical parameters were lower in healthy sites than inflamed sites ($P < .001$ for PI; $P = .035$ for PD; $P < .001$ for GBTI). PISF volume was significantly greater in inflamed sites compared to healthy sites ($P < .001$). At all of the experimental sites, detectable levels of nitrite were available. However, the mean total nitrite level was lower in healthy sites (0.1421 nmol) than in inflamed sites (0.1615 nmol) ($P = .032$). On the contrary, nitrite concentration was higher in healthy sites than in inflamed ones ($P < .001$) (Table 1). PISF volume was negatively correlated with nitrite concentration at both noninflamed ($r = –0.891$, $P < .001$) and inflamed ($r = –0.896$, $P < .001$) peri-implant sites, while there was a positive correlation between the PISF volume and PD in both healthy ($r = 0.439$, $P = .002$) and inflamed sites ($r = 0.395$, $P = .004$). Total nitrite levels and nitrite concentration did not demonstrate any correlation with the clinical parameters ($P > .05$).

Table 2 displays the data for the recorded clinical parameters, PISF volume, total nitrite levels, and nitrite concentration for the implants as classified by level of gingival inflammation. All clinical parameters except for PD were significantly lower for healthy sites than for slightly inflamed sites ($P < .001$ for PI; $P = .015$ for GBTI). All clinical parameters were significantly lower for healthy sites than for moderately/severely inflamed ones ($P < .001$ for PI; $P = .011$ for PD; $P < .001$ for GBTI). Further, PISF volume was lower in noninflamed peri-implant sites compared to slightly and moderately/severely inflamed sites ($P < .001$ for both).

Total nitrite levels and nitrite concentration presented a different pattern in regard to severity of inflammation. Total nitrite levels were lower in healthy sites (0.1421 nmol) than in slightly inflamed (0.1485 nmol) or moderately/severely inflamed sites (0.1728 nmol). However, the difference was only significant between healthy and moderately/severely inflamed

**Table 1 Statistical Analysis of Clinical Parameters and Nitrite Levels of Healthy (GI = 0) and Inflamed (GI ≤ 1) Peri-implant Sites**

| | Healthy (n = 46) | | Inflamed (n = 54) | | | |
|---|---|---|---|---|---|---|
| | Mean ± SD | Median (min–max) | Mean ± SD | Median (min–max) | z | *P* |
| PI | 0.1250 ± 0.380 | 0.00 (0.00–2.00) | 1.250 ± 1.106 | 1.00 (0.00–3.00) | –5.456 | < .001 |
| PD | 1.6522 ± 0.549 | 1.8750 (1.00–2.75) | 2.004 ± 0.801 | 2.00 (1.00–3.75) | –2.110 | .035 |
| GBTI | 0.2557 ± 0.572 | 0.00 (0.00–2.25) | 0.7212 ± 0.687 | 0.75 (0.00–2.25) | –3.676 | < .001 |
| PISF volume (μL) | 0.2117 ± 0.121 | 0.1900 (0.02–0.54) | 0.4243 ± 0.235 | 0.3900 (0.09–1.10) | –5.032 | < .001 |
| Total nitrite level (nmol) | 0.1421 ± 0.039 | 0.1358 (0.08–0.23) | 0.1615 ± 0.045 | 0.1442 (0.08–0.30) | –2.139 | .032 |
| Nitrite concetration (nmol/μL) | 0.9997 ± 0.848 | 0.6763 (0.19–4.67) | 0.5072 ± 0.318 | 0.4427 (0.15–1.60) | –4.157 | < .001 |

sites ($P = .013$). On the contrary, nitrite concentration was significantly higher in healthy sites than in slightly inflamed ($P = .001$) or moderately/ severely inflamed sites ($P < .001$). PISF volume demonstrated a negative correlation with nitrite concentration for healthy ($r = –0.891$, $P < .001$), slightly inflamed ($r = –0.928$, $P < .001$) and moderately/severely inflamed ($r = –0.837$, $P < .001$) sites. PISF volume demonstrated a positive correlation with PD for healthy ($r = 0.409$, $P = .005$) and moderately/severely inflamed ($r = .452$, $P = .018$) sites, but not for slightly inflamed sites ($P > .005$). Total nitrite level and nitrite concentration for all sites did not show any significant correlation with clinical parameters except for the correlation with the PD of healthy ($r = –0.426$, $P = .003$) and moderately/severely inflamed ($r = –.501$, $P = .008$) sites.

### Analysis of Implants Grouped by Loading Protocol

Table 3 shows data for the recorded clinical parameters, PISF volume, total nitrite levels, and nitrite concentration of the sites of EL and DL implants. Mean PI was significantly higher for DL ($U = 8.000$, $P = .01$) and EL ($U = 141.000$, $P < .001$) in the inflamed sites compared to noninflamed sites. Mean GBTI was also significantly higher in inflamed sites of EL implants compared to healthy sites ($U = 132.500$, $P = .001$). Further, mean PISF volume in inflamed sites of DL ($U = 3.500$, $P = .001$) and EL ($U = 85.000$, $P < .001$) implants was significantly higher than healthy sites. Higher mean total nitrite levels were observed in inflamed sites (0.1798 nmol) compared to healthy ones (0.1310 nmol) for DL as well as for EL (0.1584 nmol and 0.1501 nmol, respectively). However, nitrite concentration was lower in inflamed sites compared to noninflamed sites for DL ($U = 5.000$, $P = .003$) and EL implants ($U = 97.500$, $P < .001$). Moreover, in regard to the healthy sites, a significantly higher PISF volume ($U = 27.500$, $P = .001$) was found for EL compared to DL implants, and the nitrite concentration was significantly lower in healthy EL sites compared to healthy DL sites ($U = 28.500$, $P = .001$). In regard to healthy implant sites, relatively higher total nitrite levels were observed in EL sites ($P = .302$), while in inflamed sites DL implants generally provided relatively higher PISF nitrite levels ($P = .302$). There was not a constant correlation between the parameters; however, PISF volume demonstrated a constant negative correlation with nitrite concentration for DL implants with healthy ($r = –0.964$, $P < .001$) or inflamed ($r = –0.714$, $P = .047$) sites and EL implants with healthy ($r = –0.822$, $P < .001$) or inflamed ($r = –.835$, $P = .0001$) sites.

### Evaluation of Mean Total Nitrite Levels During 9 Months of Follow-up

The nitrite levels of DL and EL implants with healthy peri-implant sites are shown on the diagram in Fig 1. The total nitrite level of DL implant sites decreased between 3 and 6 months postloading, then increased between months 6 and 9. For EL implants, a decrease in the total nitrite level was observed between months 1 and 3, while an increase was noted between months 3 and 9.

Figure 2 demonstrates the total nitrite levels of DL and EL implants with inflamed peri-implant sites. In regard to inflamed sites, a similar pattern of reduction in nitrite level for both types of loading was noticed following the onset of loading. DL implants with inflamed peri-implant sites showed an increase between months 6 and 9 postloading, where EL implants demonstrated an increase between 3 and 9 months of follow-up.

**Table 2 Statistical Analysis of Clinical Parameters and Nitrite Levels of Healthy (GI = 0), Slightly Inflamed (GI ≤ 1), and Moderately/Severely Inflamed (GI > 1) Peri-implant Sites**

| | | Healthy (n = 46) | | Slightly Inflamed (n = 26) | | Moderately/severely inflamed (n = 28) | | Healthy vs slightly inflamed sites | | Healthy vs moderately/severely inflamed sites | | Slightly inflamed vs moderately/severely inflamed sites | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\chi^2$ | Mean ± SD | Median (min–max) | Mean ± SD | Median (min–max) | Mean ± SD | Median (min–max) | U | *P* | U | *P* | U | *P* |
| PI | 34.308 | 0.1250 ± 0.380 | 0.00 (0.00–2.00) | 0.8646 ± 0.997 | 0.75 (0.00–3.00) | 1.5804 ± 1.103 | 2.00 (0.00–3.00) | 311.000 | < .001 | 200.500 | < .001 | 216.500 | .023 |
| PD | 6.174 | 1.6522 ± 0.549 | 1.8750 (1.00–2.75) | 1.9063 ± 0.852 | 2.00 (1.00–3.75) | 2.0893 ± 0.758 | 2.00 (1.00–3.75) | 487.000 | .404 | 420.000 | .011 | 273.500 | .243 |
| GBTI | 15.503 | 0.2557 ± 0.572 | 0.00 (0.00–2.25) | 0.5625 ± 0.604 | 0.50 (0.00–1.75) | 0.8571 ± 0.734 | 0.75 (0.00–2.25) | 368.000 | .015 | 323.500 | < .001 | 257.000 | .133 |
| PISF volume (µL) | 25.477 | 0.2117 ± 0.121 | 0.1900 (0.02–0.54) | 0.4044 ± 0.221 | 0.3300 (0.13–0.90) | 0.4421 ± 0.250 | 0.4450 (0.09–1.10) | 253.000 | < .001 | 249.000 | < .001 | 324.500 | .649 |
| Total nitrite level (nmol) | 6.696 | 0.1421 ± 0.039 | 0.1358 (0.08–0.23) | 0.1485 ± 0.029 | 0.1442 (0.10–0.22) | 0.1728 ± 0.054 | 0.1612 (0.08–0.30) | 493.000 | .323 | 440.000 | .013 | 269.000 | .104 |
| Nitrite concentration (nmol/µL) | 17.280 | 0.9997 ± 0.848 | 0.6763 (0.19–4.67) | 0.4891 ± 0.271 | 0.4828 (0.15–1.01) | 0.5233 ± 0.359 | 0.4382 (0.17–1.60) | 308.000 | < .001 | 318.500 | < .001 | 338.000 | .831 |

**Table 3 Statistical Analysis of Clinical Periodontal Parameters and Nitrite Levels of DL and EL Implants**

| | | DL | | | | EL | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Healthy | | Inflamed | | Healthy | | Inflamed | |
| | $\chi^2$ | Mean ± SD | Median (min–max) | Mean ± SD | Median (min–max) | Mean ± SD | Median (min–max) | Mean ± SD | Median (min–max) |
| PI | 22.447 | 0.00 ± 0.00 | 0.00 (0.00–0.00) | 0.9375 ± 0.777 | 1.00 (0.00–2.00) | 0.1346 ± 0.325 | 0.00 (0.00–1.00) | 1.4479 ± 1.237 | 2.00 (0.00–3.00) |
| PD | 2.088 | 1.5313 ± 0.661 | 1.2500 (1.00–2.75) | 1.5625 ± 0.477 | 1.6250 (1.00–2.00) | 1.6058 ± 0.491 | 1.7500 (1.00–2.25) | 1.9375 ± 0.876 | 2.00 (1.00–3.75) |
| GBTI | 24.893 | 0.2187 ± 0.525 | 0.00 (0.00–1.50) | 1.1250 ± 0.654 | 1.2500 (0.00–2.25) | 0.0577 ± 0.248 | 0.00 (0.00–1.25) | 0.7708 ± 0.706 | 0.7500 (0.00–2.00) |
| PISF volume (µL) | 31.282 | 0.1012 ± 0.064 | 1.1100 (0.02–0.19) | 0.3600 ± 0.170 | 0.3300 (0.16–0.64) | 0.2342 ± 0.103 | 0.2200 (0.06–0.44) | 0.5070 ± 0.264 | 0.4600 (0.15–1.10) |
| Total nitrite level (nmol) | 3.648 | 0.1310 ± 0.321 | 0.1400 (0.08–0.17) | 0.1798 ± 0.059 | 0.1739 (0.11–0.30) | 0.1501 ± 0.040 | 0.1379 (0.08–0.23) | 0.1584 ± 0.044 | 0.1442 (0.08–0.25) |
| Nitrite concentration (nmol/µL) | 28.541 | 1.9555 ± 1.394 | 1.4530 (0.80–4.67) | 0.5895 ± 0.271 | 0.5514 (0.25–1.01) | 0.7797 ± 0.445 | 0.6531 (0.37–1.89) | 0.3862 ± 0.213 | 0.3614 (0.15–0.96) |

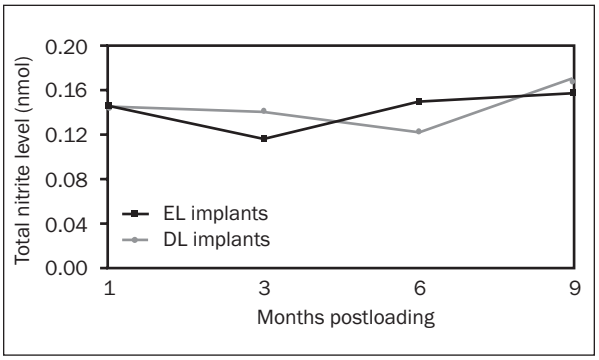


Fig 1 Mean total nitrite levels of DL and EL implants with healthy peri-implant sites during 9 months of follow-up.

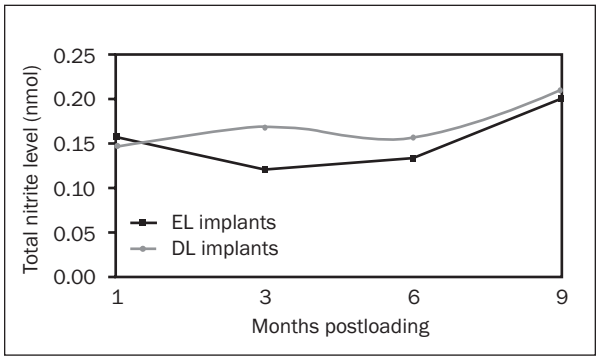


Fig 2 Mean total nitrite levels of DL and EL implants with inflamed peri-implant sites during 9 months of follow-up.

## DISCUSSION

NO has been considered an important molecular signal in a wide variety of tissues and may play a significant role as a cytotoxic mediator of the nonspecific immune response, with both harmful and beneficial effects.[2–4] While the constitutive forms, endothelial NOS (eNOS) and neuronal NOS (nNOS), produce low NO concentrations for a short period of time, iNOS is expressed in response to inflammatory stimuli, resulting in greater amounts of NO production for a long time period.[2,3,18] Unlike the constitutive forms, the activity of iNOS is not regulated, and therefore much of the nitrite of body fluids is formed from oxidation of NO produced by iNOS. Previous studies have indicated the presence of iNOS in gingivitis and in chronic and aggressive periodontitis.[7,11,16–19,40] Briefly, immunohistochemical analysis in cases of human periodontal disease, either gingivitis or chronic periodontitis, has demonstrated a significant increase in the number of iNOS positive cells, predominantly polymorphonuclear cells, in relation to clinically healthy gingival tissues containing discrete inflammatory infiltrate.[18]

It has been suggested that the relative expression of interleukin-6 and iNOS mRNA is significantly higher in patients with chronic periodontitis than in healthy individuals.[16] In LAP, immunohistochemical staining demonstrated that macrophages expressed high levels of iNOS, which can damage the periodontal tissues.[7] Another immunohistochemical study underlined the importance of macrophages in iNOS production in periodontitis; the other important source given was endothelial cells in the blood vessels.[17] Lappin and colleagues[17] further suggested that the diseased tissue had a greater infiltration of iNOS-expressing cells and more pronounced endothelial cell expression of this enzyme than clinically healthy gingiva, which supported the results of Matejka and coworkers.[17,41] It was also stated that treatment with iNOS-inhibiting drugs could block the production of NO or its effects, where periodontally involved human gingival fibroblasts expressed high levels of iNOS and where NO synthesis was induced by proinflammatory cytokines such as tumor necrosis factor, interleukin-1, and interferon.[40] All of these studies noted that NO has a pivotal role in subjects with gingivally or periodontally compromised teeth.

Because of the reactivity of NO and its short life, direct measurements of NO from body fluids have been thought hard to perform.[42] In contrast, measuring nitrite was introduced as much easier and straightforward.[42] Both the reduction of nitrate and NO oxidation cause nitrite production. Thus, in the present study, the nitrite levels of PISF using the Griess reaction were analyzed.[34] The findings based on this stable end product of NO oxidation[42] support the concept that NO production increases with inflammation; PISF nitrite levels were higher at inflamed implant sites compared to healthy ones.[7,11,16-19,40]

With respect to the severity of clinical gingival inflammation, the total nitrite level of slightly inflamed sites demonstrated only a trend of increase compared to healthy sites. However, the nitrite level was significantly increased in moderately/severely inflamed sites compared to healthy sites. Based on the present results in regard to total nitrite levels, there is agreement with previous studies regarding the action of NO in periodontal sites with gingival or periodontal disease.[7,16–18,40,42] However, it may be further suggested that NO production depends on the severity of clinical inflammation and that the role of NO in inflammation is similar in dental implant sites. In contrast, the nitrite concentration in inflamed peri-implant sites was significantly lower than in healthy sites, and with respect to gingival inflammation, less nitrite concentration was found compared to healthy sites. For a given dental implant site, this contrast between the 2 modes of data presentation

suggests the volume-dependent nature of the concentration expression.[43,44] In regard to the severity of gingival inflammation, greater PISF volume was obtained from slightly and moderately/severely inflamed sites than from healthy sites, which is clearly in accordance with previous studies demonstrating a close association between increased gingival crevicular fluid volume and clinical inflammation.[45,46] Despite the high total nitrite levels, the lower nitrite concentrations found at inflamed sites may be related to the greater amounts of PISF found at these sites. As concentration expression is affected by the available PISF volume in a given site, and total nitrite levels are independent of volume, the latter seems to be a more appropriate mode of data presentation for PISF nitrite levels. A similar suggestion has been made in regard to various other gingival crevicular fluid constituents.[43,44]

Maintenance of the bone mass and the architecture, remodeling, and mechanisms that regulate the adaptive capacity of bone are complex processes that mainly result from the coordination of formation and resorption, which is mediated by the bone cells.[4,20,47] Mechanical loading modulates bone remodeling by provoking the cellular response via loading-induced flow of interstitial fluid through the lacuno-canalicular network of osteocytes, which in turn leads to an adaptation of local bone mass and architecture.[48] In vitro bone cells are highly responsive to mechanical stimuli, and cells can be influenced differently by fluid shear, tension, and compression.[49] As NOS activity in osteoblasts and osteocytes is known to be stimulated by mechanical strain,[20,47] and shear stress rapidly stimulates a cNOS isoform in osteoblasts, NO is an important autocrine/paracrine factor that may modulate bone remodeling process[20] and is suggested to be necessary for the bone's mechanically induced anabolic response.[50]

Mullender and colleagues[49] stated that mechanical forces played an important role in the regulation of bone remodeling in intact bone and bone repair, where strain applied through the substrate and fluid flow stimulated the release of signaling molecules such as NO. On the other hand, it was suggested that small amounts of NO produced by osteoblasts could act as autocrine stimulators of osteoblast growth and cytokine production,[4] while high concentrations, such as those observed after stimulation with proinflammatory cytokines, had potent inhibitory effects on osteoblast growth and differentiation.[4] Kunnel and colleagues also supported the fact that bone anabolic responses to mechanical load involved cNOS and mechanical stimulation essential for maintaining the homeostasis and architecture of bone, making NO an important regulatory molecule in bone formation and resorption induced by mechanical stimulation.[50]

Orthodontic tooth movement has been shown to share similarities with inflammation and wound healing. This adaptive remodeling process represents a concerted effort of the whole periodontium and alveolar bone.[22,23] Orthodontic studies noted that NO precursor application significantly increased tooth movements, whereas NO inhibitors reduced orthodontic tooth movements.[22] NO has been suggested to diffuse to the alveolar bone and influence the function of osteoclastic differentiation, where the osteoblast function is also modulated by NO-dependent mechanisms.[22] Shirazi and colleagues also suggested that NO played a role in bone resorption as well as formation and facilitated these phenomena during force application.[22] Mechanical strain and shear stress have been shown to cause rapid increase in the production of NO.[20,47] Osteoblasts and osteocytes under noninflammatory conditions only express the eNOS isoform, whereas mechanical loading has resulted in an increase of NO production, supporting the notion that osteocytes are the principal cells that respond to mechanical stress in bone.[4]

In the present study, the sites of DL and EL implants provided different total nitrite levels in both the presence and absence of clinical inflammation, although the differences were not statistically significant. Where the peri-implant tissue was healthy, higher total nitrite levels were observed at the sites of EL implants; where the peri-implant tissue was inflamed, higher total nitrite levels were observed at the sites of DL implants.

As the contribution of inflammation to higher NO production is not expected at noninflamed sites, the higher PISF nitrite level may be attributed to mechanical stress resulting from the loading of the EL implants. In inflamed sites, the contribution of both inflammation and loading can be considered. Although statistical analysis could not be performed, a change in the pattern of PISF total nitrite levels was observed both for EL and DL implants during follow-up, particularly with the onset of loading. In healthy peri-implant sites, a trend of reduction for a short period of time was observed (Fig 1). After 3 months of loading for EL and DL implants, respectively, both groups demonstrated a trend of increase through 9 months of follow-up. Both inflamed and noninflamed peri-implant sites (Figs 1 and 2) showed a reduction in total nitrite level followed by an increase between baseline and the 9-month follow-up. As NO is suggested to be involved in both bone formation and bone resorption, and the role of NO in bone remodeling is suggested to be concentration-dependent,[4,49] the need for a tight regulation of NO around

dental implants may be assumed. The observed pattern of NO in the present study may be speculated to reflect the presence of a mechanism for the regulation of NO production through the process of bone repair, bone remodeling, and adaptation to the applied forces that enables the maintenance of the supporting bone around the dental implants. In regard to the higher amounts of total nitrite activity observed at the healthy sites of DL and EL implants during follow-up (Figs 1 and 2), again the contribution of both the inflammatory process and loading can be considered at these sites.

Thus, besides the presence and severity of inflammation, there seems to be a potential for loading to contribute to the NO metabolism around dental implants. Previous studies using in vitro study designs or orthodontic force application models also suggest an increase in NO production in the bone remodeling and bone repair processes with mechanical loading, mechanical strain, and shear stress and increases in the NO-dependent activities of osteoblast and osteoclasts.[4,20,22,47–50] There is considerable evidence that fluid flow–induced shear stress rapidly stimulates NO release from osteoblasts, preosteoclast-like cells, and osteocytes in response to mechanical strain or stress during remodeling of the bone[20,21] and that fluid flow–induced shear stress NO production plays a primary role in bone maintenance and remodeling.[20] Thus, the findings of the present study using a dental implant model may generally support previous studies that suggest a role for NO in bone metabolism,[22,23] and emphasize the impact of force application on NO metabolism and bone remodeling.[4,20,22,47–50] Furthermore, as gingival crevicular fluid volume may also be affected by mechanical stimulus, the higher PISF volume observed for EL implants compared to DL implants may also be attributed to the loading of dental implants, indicating the potential impact of mechanical forces on both the quantity and quality of PISF.

However, follow-up figures of the present study need specific caution because of the limited number of sampling sites. Further studies are needed to clarify the distinct action of NO in bone metabolism. This ongoing longitudinal study is expected to provide further evidence for clarification of the relationship between loading of dental implants and NO and bone metabolism.

## CONCLUSIONS

Using a dental implant study model and PISF nitrite levels, the findings of the present study support increased production of NO with the development of inflammation as a result of the intensity of the migrating inflammatory cells to the implant site and further demonstrate dependence of NO metabolism on the severity of clinical inflammation. Loading of dental implants also seems to have the potential to influence the NO metabolism, suggesting a role for NO in bone repair and remodeling around dental implants and the presence of a tight control of NO production that enables the maintenance of supporting bone.

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