Original Article - Prospective Study

Effects of Nitric Oxide Supplementation on the Levels of Interleukin-6 in Saliva after Dental Implant Placement - A Prospective Study

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Abstract

Introduction: The purpose of the study was to evaluate and compare the levels of salivary Interleukin-6 (IL-6) before and after the placement of dental implants in patients who are supplemented with nitric oxide (NO). **Materials and Methods:** The study comprised 34 patients, divided into control and study groups (17 in each group). The control group was given a placebo and in the study group, nitric oxide supplement was prescribed, whereas the control group received a placebo. Saliva samples were taken before placement of dental implants, first and third day after the implant placement. The levels of salivary IL-6 were assessed using an enzyme-linked immunosorbent assay test. **Results:** Statistical analysis showed a significant *P* value (<0.05) with respect to IL-6 levels on first and third days after placement of dental implants. Salivary IL-6 levels in the study group declined significantly. On day three, the IL-6 values for the control and study groups were 0.0639 and 0.0443, respectively. Within the groups, it was observed that there was a significant decrease in IL-6 values from day one to day three. **Discussion:** The levels of salivary IL-6 reduced from day one to day three more significantly and consistently in patients prescribed with NO supplements post-dental implant placement, suggesting better resolution of inflammation.

Keywords: Dental implants, inflammation, nitric oxide, pro-inflammatory cytokines

Introduction

Dental implant therapy involves a process where osteotomy is performed, creating a wound to receive a dental implant. One of the major processes before wound healing is the process of inflammation. At the cellular level, many inflammatory changes do occur, which include the production of pro-inflammatory cytokines such as interleukin (IL)-6, IL-10 and tumour necrosis factor (TNF)-∝.[1] Implantation of any biomaterial can instantly activate the innate immune system, which leads to the development of an acute inflammatory state that marks the initial step of tissue repair. This initial inflammatory state aids in bone healing, but chronic inflammation or fibre encapsulation could result from prolonged inflammation and could boost the activity of osteoclasts, which would control the entire osseointegration process.[2] After implantation, from the 3rd day, the inflammatory response begins to reduce. The ability of endothelial nitric oxide synthase (eNOS) to extract nitric oxide (NO) through the L-arginine-NOS pathway in the vascular endothelium to restore homeostasis is one of the major biological processes for the resolution of the inflammatory process. NO has a substantial role in regulating the blood flow for vascular integrity.[3]

NO has shown its significant role in numerous biological, functional and inflammatory processes, including vasodilation, modulation of neurotransmission and suppression of microbial and tumour cell growth.[4] The amino acid L-terminal arginine's guanidino nitrogen is converted by the NO synthase (NOS) enzymes into NO, with the by-product L-citrulline. There are

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three types of NOS: an inducible form (iNOS or NOS2), an endothelial form (NOS3) and a neuronal form (NOS1).

Dietary nitrate enters the bloodstream through the L-arginine-NOS pathway after being absorbed in the small intestine. However, the salivary glands absorb and create around 25% of the circulating nitrate, which is then transformed into nitrite by commensal bacteria in the mouth.[5] On consumption, nitrite is taken in by the digestive system and further reduced to NO and other bioactive nitrite intermediates in the blood and tissues by enzymatic and non-enzymatic processes. The nitrate-nitrite-NO pathway's nitrite reduction to NO, in contrast to NOS-dependent NO generation, is oxygen independent and advantageous during tissue ischaemia.[6] IL-6, which is primarily produced by immune cells and which has been shown to play a significant role in promoting the expression of other inflammatory mediators and cytokines involved in osteoclast differentiation and activation, may be a major factor in the increased severity of inflammatory bone resorption.[7]

Studies have been performed regarding the quantitative analysis of NO levels around dental implants and its effect on inflammation and implant stability. NO supplements are widely used as an adjunctive therapy in cardiovascular patients, pregnancy, post-surgery to reduce inflammation, etc.[8] In dental implant surgical procedures, the use of NO supplements has not been stated and hence, this study focuses on the effect of NO supplements on salivary IL-6 levels after placement of dental implant. The null hypothesis is that there is no effect of NO supplement on salivary IL-6 levels after placement of dental implants.

Materials and Methods

In this clinical study, patients seeking prosthodontic rehabilitation for missing teeth were included. The Ethical Committee (MRIIRS/MRDC/FDS/IEC/2020/10) approved the study protocol, data collection forms and informed consent form. The procedures adhered to the ethical guidelines of the Declaration of Helsinki. Sample size estimation was done by using G Power software (version 3.0, Erdfelder, Faul, and Buchner, 1996). The results obtained were subjected to t test and mean difference between the two independent means. A total sample size of 34 (17 in each group) was adequate for an alpha of 0.05, power of 80%, and confidence interval of 95% (assessed for difference in IL-6 level before and after implant placement from a similar study).

Patients ranging from 18-45 years of age, one missing tooth, with adequate bone volume to accommodate the implant and willing to undergo the treatment were included in the study. Smokers, patients with precancerous or other neoplastic lesions, bony disorders, inflammatory conditions such as rheumatoid arthritis and gout, a history of immunosuppressive, anticoagulant or anticancer drugs within three months of study, medications affecting salivary secretions, pregnant and lactating females were excluded from the study.

Mandatory blood investigations before surgical procedure were carried out. After the preparation of the implant site, full-thickness flap was reflected followed by sequential drilling depending on the selected implant size. Implant was placed in the prepared osteotomy and implant stability quotient of more than 60 were selected for the study. Suturing was done with 3-0 sutures. Group I (Control group) patients were prescribed a placebo, while the patients in the Group II (Study group) were prescribed NO supplement along with anti-inflammatory and antibiotic drugs for both the groups. Beetroot supplements were used as NO supplement (1000 mg once daily). Saliva sample was taken at baseline, i.e., before placement of dental implant, 1st and 3rd day after placement of implant. One hour before the test, the patient was asked to abstain from all food and liquid consumption (water was an exception). The patient was instructed to repeatedly rinse their mouths with deionised (distilled) water before taking a 5-min break. Disposable needle less syringes were used for saliva collection. Saliva was collected from lingual vestibule. 1–2 mL of collected saliva was poured into coded capped Eppendorf tubes and were stored at −20° Celsius. Further, the saliva samples were used to evaluate the IL-6 levels by using an enzyme-linked immunosorbent assay (ELISA) kit.

For the estimation of salivary interleukin-6, the required number of microwells were arranged on the microwell plate, 100 µL of calibrators, controls (C1–C6, D, E) and unknowns to microwells (A) were dispensed and microwell film seal was used to cover the microwells. The microwell was shaken for 1 h and 30 min at 480 RPM. Using 300 mL of ×1 Wash Buffer preparation, each well was washed five times. Then, 100 mL of horseradish peroxidase (HRP) conjugate (B) was added to each well, and was incubated for an hour at room temperature. Each well received an additional five washings with 300 mL of ×1 Wash Buffer preparation, 100 mL of 3,3',5,5'-Tetramethylbenzidine (TMB) Solution (TS) and 30 min of incubation at room temperature and away from light. Each well received 50 L of Stop Solution, and the levels of IL-6 were measured using an ELISA reader.

Results

SPSS software (IBM®SPSS®Statistics 24.0, Chicago), between control and study groups at different time intervals, which showed a significant *P* value (<0.05) with respect to IL-6 levels on the 1st and 3rd day after placement of dental implants. The data were non-parametric in nature, and hence, for intergroup comparison, Mann–Whitney *U*-test was applied, and for intragroup comparison, Wilcoxon signed-rank test was applied.

In Tables 1-3, intergroup comparisons of the salivary IL-6 levels at baseline, 1st and 3rd days have been discussed. Significant and consistent decrease in the levels of salivary IL-6 was observed with respect to the study group (Group II), from 0.05571 (baseline) to 0.0443 (3rd day). While in the control group (Group I), an increase in the levels of salivary IL-6 was observed from 0.05588 (baseline) to 0.0639 (3rd day).

Significant *P* value at $P < 0.05$ was obtained with respect to 1st and 3rd days. Tables 4 and 5 show the result after intragroup comparison of salivary IL-6 levels from baseline to 1st day, 1st day to 3rd day and from baseline to 3rd day. In the study group, salivary IL-6 levels reduced significantly from 1st day to 3rd day ($P < 0.05$). In the control group, an increase in the salivary IL-6 levels was observed consistently from baseline to 3rd day ($P < 0.05$) and also from 1st day to 3rd day ($P < 0.05$).

Discussion

Numerous crucial elements, such as pro-inflammatory cytokines, anti-inflammatory cytokines, macrophages, NO molecules and various other vascular factors control the transition of inflammation to osseointegration.[9,10] NO is a free radical, produced either by NOS or through Nitrate-Nitrite-NO pathway, which is found at the site of inflammation, i.e. around the dental implant surface.[11] In our study, the control group's salivary IL-6 levels significantly increased from 0.05588 (baseline) to 0.0639 (3rd day) following the implantation of dental implants. Similar findings were obtained in a study performed by Tomeleri *et al*.,[12] in which an increase in the levels of IL-6 (2.8 ± 0.6 3.1 ± 0.9) was observed in cases of inflammatory conditions. Pro-inflammatory cytokine levels in the gingival crevicular fluid (GCF) around dental implants were compared in a study by Negahdari *et al*.[13] The results showed lower levels of IL-1 (titanium = 20.42 ± 6.48; ceramic = 13.05 4.59) and higher levels of IL 6 (titanium = 46.17 ± 1.25; ceramic = 25.11 ± 3.38) in the GCF around ceramic abutments. Al Ghazal *et al*.,[14] evaluated different debridement methods that preserved and improved the soft tissues around implants over the course of a year. The results showed a statistically significant correlation ($P = 0.05$) between the levels of IL-6 in the deepest pocket around these implants and the proportion of bleeding on probing per implant. According to a study by Abduljabbar *et al*.,[15] there is a correlation between total salivary IL-1, IL-6 and TNF-α levels and patients with and without peri-implantitis, peri-implant clinical and radiological features and self-reported discomfort. The results showed that peri-implantitis participants had considerably greater levels of IL-1, IL-6 and TNF-α than peri-implantitis-free participants. Sayardoust *et al*.[16] concluded that IL-6 was one of the consistent pro-inflammatory cytokines found in the peri-implant tissues, with stimulating effects on both osteoclasts as well as osteoblastic cells. In a study by Marcello-Machado *et al*.,[17] it was found that IL-6 is a key indicator of inflammatory processes, is considered to activate osteoclasts, is continually released by particle-stimulated cells, and has a significant role in the pathogenesis of osteolysis. Isler *et al*.[18] proved the presence of pro-inflammatory cytokine IL-6 in the oral fluids, for example, saliva, GCF, peri-implant sulcular fluid (PISF), etc., which yields pertinent information regarding the progression of oral inflammatory conditions.

The understanding of the "nitrate-nitrite-NO pathway" led to the finding that dietary (inorganic) nitrate has significant vascular effects.[19] It has been evidenced that dietary nitrates have a number of positive vascular effects, including management of blood pressure, impeding platelet aggregation post-surgeries, a key role in preventing endothelial dysfunction and improving exercise capacity in both healthy people and those with

Table 1: Comparison of two study groups with respect to interleukin-6 levels at baseline

Groups	Mean±SD	Median	Minimum	Maximum
Group I (study)	0.05571±0.008	0.056	0.037	0.067
Group II (control)	0.05588±0.014	0.061	0.004	0.066
Mann–Whitney *U*		169.5		
P		0.394		

Mann–Whitney *U*-test applied; *P* value non-significant at $P>0.05$.
SD: Standard deviation

Table 2: Comparison of two study groups with respect to interleukin-6 levels 1 day after implant placement

Groups	Mean±SD	Median	Minimum	Maximum
Group I (study)	0.05659±0.016	0.056	0.029	0.092
Group II (control)	0.06106±0.0039	0.062	0.050	0.067
Mann–Whitney *U*		203.5		
P		0.041		

Mann–Whitney *U*-test applied; *P* value significant at $P<0.05$.
SD: Standard deviation

Table 3: Comparison of two study groups with respect to interleukin-6 levels 3 days after implant placement

Groups	Mean±SD	Median	Minimum	Maximum
Group I (study)	0.0443±0.0157	0.043	0.019	0.083
Group II (control)	0.0639±0.0043	0.063	0.057	0.074
Mann–Whitney *U*		270.0		
P		0.0001		

Mann–Whitney *U*-test applied; *P* value significant at $P<0.05$.
SD: Standard deviation

Table 4: Intragroup comparison in control group of the mean values at different time intervals

Time interval (days)	Mean±SD	*P*
Baseline to 1	−0.0052±0.0114	0.079
1–3	−0.00288±0.0038	0.007*
Baseline to 3	−0.00881±0.01419	0.033*

**P*-value significant at $P<0.05$. Wilcoxon signed-rank test applied.
SD: Standard deviation

Table 5: Intragroup comparison in the study group of the mean values at different time intervals

Time interval (days)	Mean±SD	*P*
Baseline to 1	−0.00088±0.0127	0.779
1–3	0.01223±0.0175	0.011*
Baseline to 3	0.01135±0.0163	0.011*

**P*-value significant at $P<0.05$. Wilcoxon signed-rank test applied.
SD: Standard deviation

Table 6: Studies regarding nitric oxide supplements and their effect on cytokine levels

Reference	Intervention	Nitrite dose	Nitrate dose	Positive effects	Negative effects
Sindler *et al.* (2011)[22]	Sodium Nitrite	33.5mg/L		Aorta: ↓IL-1β; ↓IL-6; ↓TNF-α	-
Sindler *et al.* (2015)[23]	Sodium Nitrite	33.5mg/L		Aorta: ↓ IL-6	No effect on circulating IL-6
Ohtake *et al.* (2017)[24]	Sodium Nitrite	33.5mg/L		↓IL-6; ↓ TNF-α; ↓ MCP-1	-
Li *et al.* (2016)[25]	Sodium Nitrate		15, 30, and 6 mg/kg of BW/dL	↓ CRP; ↓TNF-α; ↓IL-6; ↓ ET-1	-
Ashor *et al.* (2016)[26]	Potassium Nitrate	4.3 mg/kg of BW		↓ E-selectin; ↓ P-selectin; ↓ IL-6	No change to ICAM -3

↓: Significant reduction in the values, BW: Body weight, ICAM-3: Intercellular adhesion molecule-3

peripheral artery disease.[20,21] The role of NO supplementation and its impact on cytokine levels are discussed [Table 6].[22-26] Sodium nitrate used in the above-mentioned studies was used as a NO supplement which was added to the drinking water. Beetroot is known to have the maximum amount of nitrate content, followed by Spinach.[27]

The null hypothesis of the study was rejected. A significant and consistent reduction in the levels of salivary IL-6, from 0.05571 (baseline) to 0.0443 (3rd day), was observed in the study group who were prescribed NO supplements. Tözüm *et al*.[28] performed an investigation into the possible impact of clinical status, level of inflammation and loading protocol on NO metabolism around dental implants. The findings demonstrated that NO plays a role in the peri-implant inflammatory process. In addition, loading may have an effect on NO metabolism, which raises the possibility of remodelling and adaptability of the bone around dental implants. In peri-implant tissues of titanium and zirconium oxide healing abutments, Degidi *et al*.[29] conducted a comparative immunohistochemical evaluation of the expression of vascular endothelial growth factor (VEGF) and NOS and inflammatory biomarkers. NOS1, NOS3 and VEGF variations between low and high levels were statistically significant ($P = 0.0001$). The results showed that the expression of NOS1, NOS3 and VEGF was highest in the titanium group (36.517.3) and lowest in the zirconium oxide group (22.114.4). The findings suggested that NOS plays an active role in the resolution of inflammation by having a direct impact on the concentrations of inflammatory infiltrate. An investigation was conducted by Tözüm *et al*.[30] to determine if the marginal bone levels, resonance frequency analysis (RFA) values and the PISF's nitrite content could be interrelated with the effects of the loading protocol in unsplinted dental implants. It was found that the NO biomolecule affects bone cell activity, bone maintenance and bone remodelling by being implicated in the inflammatory process of peri-implant soft tissues and bone turnover, anabolic responses and bone resorption. NO production around dental implants is significantly influenced by the loading pattern and showed varied patterns of nitrite formation in PISF.

NO shows a biphasic effect on the bone remodeling process, as it is produced by osteoblasts constitutively, which acts as a stimulator for the growth of osteoblasts and also in the secretion of cytokines. According to Loi *et al*.,[31] 72 h of an inflammatory state is necessary to forecast normal osteogenesis, and starting on the 3rd day after implantation, the osteoclastic effect must diminish to indicate that osteogenic differentiation is being positively regulated. According to Zhao *et al*.,[32] NO is a second messenger and neurotransmitter that dominates the signalling process during bone remodelling by activating the cAMP/PKA, cGMP/PKG and MAPK pathways. NO levels are reduced when inflammatory or cytokine signals are eliminated. The combination of plasmids eNOS transfection, L-arginine supplementation and calcium ions addition can be a capable therapeutic practice, according to Zhang *et al*.,[33] NO is a molecule that aids in the homeostasis mechanism for the resolution of inflammation. When NO supplements are used post-implant placement, the concentration of NO increases at the site of surgery. In the study group, IL-6 levels reduced from baseline to day three and the rate of reduction was higher (mean difference = −0.00881) as compared to the control group (mean difference = 0.01135).

The limitation of the study is that only the objective evaluation and correlation of salivary IL-6 is executed in the current study. For a better understanding of the effect of NO supplement, a clinical study evaluating the subjective sign and symptoms along with objective analysis of other biomarkers can be designed. Recent advancements in the field of dental implants have studies in progress regarding surface treatment of titanium with NO.[34] The clinical trials of such implants can help us in formulating a treatment protocol for longevity and predictive results.

Conclusion

NO has a positive effect on inflammation as it accelerates the process of transformation from inflammatory to osseointegration state. Based on our results, an enhanced rate of reduction in the inflammatory cytokine levels was observed in the patients who were prescribed NO supplements. Pro-inflammatory cytokines are known to be osteoclastic activators, and hence the action of NO supplements accelerates the process of transformation from inflammatory state to osseointegration state. And hence, NO supplement can play a vital role in the process of wound healing and successful osseointegration.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published

and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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