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Evaluation of nitric oxide levels in chronic periodontitis patients treated with initial periodontal therapy and probiotic food supplements: a double blind, randomized controlled clinical trial

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ABSTRACT

The aim of the present study was to analyse the nitric oxide (NO) levels in gingival crevicular fluid (GCF) of patients with chronic periodontitis (CP) treated with probiotic-containing food supplements as an adjunct to initial periodontal therapy (IPT). The present study was a randomized, double blind clinical trial conducted in Yeditepe University Dental Hospital, Istanbul, Turkey. Thirty-six CP patients, with ≥2 nonmolar teeth in each quadrant with probing depth (PD) of 5–7 mm at proximal sites and radiologically detectable horizontal alveolar bone loss were randomized into two treatment groups. The test group received IPT + probiotic-containing food supplements, whereas the control group received IPT + placebo. GCF sampling was performed at the baseline and 3 and 6 months after treatment. The biochemical evaluation of NO in GCF was performed using the Griess colourimetric method. Statistical analysis was performed by using statistical software. Significant reductions in GCF volume and GCF NO levels were detected in each group after the treatments ($p < 0.05$). Intergroup comparison of the NO levels revealed statistically significant differences in favour of the test group at both 3 and 6-month evaluation periods ($p < 0.05$). Adjunctive usage of probiotic comprising food supplements to IPT yielded significant reduction of the NO levels compared to the control for up to 6 months of follow-up. The present study is registered at Thai Clinical Trials Registry (TCTR identification number: TCTR20171114003).

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Introduction

Nitric oxide (NO), which is formed by the oxidation of the amino acid arginine, is an uncharged molecule and reactive free radical [1, 2]. NO is essential for various physiological processes, such as homeostatic functions (including vasodilatation, inhibition of platelet adhesion and aggregation, regulation of vascular tone, and neurotransmission), host defense against infectious agents (such as bacteria, fungi, and parasites) and tumour cell killing [2, 3]. As an uncharged molecule, NO passes freely across the cell membrane, inducing cell-to-cell communication [4]. Unlike other intercellular messengers, NO shows no binding capability to receptors. Its half-life is in the order of seconds, and its effects are transient and local [5]. Although NO is important in host defense and homeostasis, it also causes harm and has been associated with the pathogenesis of numerous inflammatory and autoimmune diseases [6–8]. NO is produced by the NO synthase (NOS) enzyme through the oxidation of L-arginine. Three distinct NOS isozymes exist in mammalian tissues: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). nNOS and eNOS are constitutively expressed in the organism, whereas iNOS is expressed only in response to inflammatory stimuli [9]. The NO produced by NOS is easily oxidised to nitrite (NO_2) and can be further oxidised to nitrate (NO_3). In biological fluids, the total levels of NO_2 and NO_3 are mostly used for analysing the NO synthesis [10].

Chronic periodontitis (CP) is a dental plaque biofilm associated inflammatory disease that develops due to an imbalance between biofilm microorganisms and host defense mechanisms leading to attachment and alveolar bone loss [11]. In the literature, the association between periodontal disease and systemic inflammation and

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oxidative stress gains research attention [12]. Bacteria and bacterial lipopolysaccharides and inflammatory mediators activate inducible nitric oxide synthase (iNOS) to produce NO [13]. Therefore, the systemic inflammatory response can be evaluated by measuring NO, NO_2 and NO_3 in biological fluids [14–16].

To date, numerous systematic reviews have revealed the initial periodontal therapy (IPT) as a gold standard in the treatment of CP. IPT reduces inflammation and the probing depth (PD) and increases the clinical attachment levels [17, 18]. However, the efficacy of IPT can be limited in deep pockets and furcation areas. For this reason, adjunctive treatment approaches are proposed to improve the efficacy of root surface decontamination and for the modulation of the oral biofilm composition [19].

Probiotics are food supplements, which used as adjunct to IPT, are reported to modulate host defenses, including both innate and acquired immune systems, by modulating the anti-inflammatory action [20]. Probiotic bacteria and their metabolites can be recognised by the epithelial and immune cells of the host [21]. Kõll-Klais et al. [22] reported that several oral *Lactobacillus* species show antimicrobial action towards periodontal pathogens. These species also exhibit high tolerance to environmental stress factors, rendering them as functional probiotic candidates for the improvement of oral health. For the management of the periodontal diseases, adjunctive probiotic usage to IPT is mostly shown to improve the periodontal clinical, microbiological and biochemical parameters [20, 23–26].

Previous studies yielded controversial results regarding the amount of NO metabolites in patients with CP [10, 16, 27–30]. In the English literature, no study has been reported about the possible adjuvant effect of probiotic supplementation to IPT on the NO levels of patients with CP. Therefore, this study aimed to analyse the NO levels in the gingival crevicular fluid (GCF) of patients with CP and treated with either IPT + probiotic comprising food supplements or IPT + placebo up to 6 months of follow-up.

Subjects and methods

Ethics statement

The study protocol was approved by the Yeditepe University Institutional Board (Decision number:128) and was conducted in full accordance with the Declaration of Helsinki, as revised in 2002. Before enrollment, the study protocol was explained in detail, and an informed consent form was obtained from all participants.

Selection of patients

The present study was a randomized, double blind clinical trial conducted in Yeditepe University Faculty of Dentistry, Istanbul. A total of 36 otherwise healthy, CP patients aged between 35 and 65 years old were included in the study. The inclusion criteria to the study were as follows: (1) patients with CP featuring moderate horizontal bone loss; (2) $\geq$ 2 nonmolar teeth in each quadrant with PD of 5 mm to 7 mm at proximal sites; (3) non-smoking; (4) reported no regular use of probiotic comprising food supplements. The exclusion criteria were as follows: (1) periodontal therapy or use of antibiotics 6 months prior to the study; (2) history of a systemic disease or the use of a medication that may affect the periodontium; (3) pregnancy and nursing; (4) allergic reactions to lactose or fermented milk products.

Randomisation

A computer-based randomisation program was used to assign the patients into two treatment groups. The test group received IPT and probiotic-containing food supplements[1] in tablet form, whereas the control group received IPT and placebo tablets.

GCF sampling

In each quadrant, two nonmolar teeth with interproximal PD of 5–7 mm with radiologically detectable horizontal bone loss were selected for sampling. The samples were obtained from the same sites at the baseline and 3 and 6 months after IPT by using sterile strips,[2] which were inserted into the gingival crevice for 30 s after isolation of the sampling site with cotton rolls and removal of supra-gingival plaque with periodontal probe. A total of eight samples per patient were obtained in each evaluation period and were pooled before NO analysis. The GCF volume was calculated with a calibrated device[3] and converted into volume (mL) by reference to the standard curve. All strips were then immediately placed in Eppendorf tubes, coded individually and stored at −80° C until NO analysis.

Clinical measurements

Clinical measurements including PD, and bleeding on probing (BoP) were obtained from 6 sites per tooth with a periodontal probe[4] used by the same calibrated examiner at the baseline and a 3- and 6-month follow-up. PD was recorded as the distance between the

gingival margin and the bottom of the periodontal pocket. BoP was assessed with the simultaneous probing, and the presence or absence of bleeding up to 30 s after probing was scored as positive (+) or negative (−) bleeding for each point.

Treatment protocol

The patients included in the study were treated at the Department of Periodontology. At the baseline, the GCF samples were obtained from both treatment groups. Supra and subgingival debridement with ultrasonic scaler and periodontal curettes were completed in two sessions with a 1-week interval under local anesthesia. Each patient was instructed about oral hygiene procedures according to their individual needs. The test group consumed 2×10^8 CFU *Lactobacillus reuteri*-containing probiotic food supplement tablets, whereas the control group consumed placebo tablets without probiotic bacteria twice a day for 3 weeks. The patients were informed to suck the tablets after tooth brushing. After completion of IPT, the GCF sampling protocol was repeated at 3 and 6 months. The probiotic and placebo tablets were placed in identical bottles and could not be separated from each other by taste, shape, nor texture. The patients were instructed not to use any probiotic food supplements during the study.

NO assay

The GCF NO levels were evaluated with the method of Miranda et al. [31] (EEA, UVU). The principle of the assay is the reduction of NO_3 by vanadium combined with detection by the acidic Griess reaction. The Griess reagents were prepared as separate solutions of *N*-(1-naphthyl) nethylenediamine dihydrochloride (NEDD) (0.1% w/v) in H_2O and sulfanilamide (2% w/v) in 5% HCl. The absorbances were measured spectrophotometrically at 540 nm. The results were calculated using the extinction coefficient of 53,000 L/(mol cm) and expressed as μmol/dL.

Statistical analysis

Statistical analysis was performed by using the statistical program GraphPad Prizma 6 (USA). Quantitative data were recorded as mean values with standard deviation (±SD). The compliance of the data with the normal distribution was evaluated using Kolmogorov–Smirnov test. Balancing of groups by age and gender was tested by Student's *t*-test and *Chi*-square test. Intragroup multiple comparisons in different evaluation periods were evaluated with one-way analysis of variance (ANOVA). Student's *t* test was used to evaluate the intergroup comparisons of the mean differences. Statistical significance was set at $p < 0.05$.

Results and discussion

Table 1 shows the demographic data and baseline parameters of the patients. No significant differences were detected between the groups regarding the baseline parameters ($p > 0.05$).

Table 2 presents the mean values of GCF volume, NO level, PD and BoP. In both groups, significant reductions were detected in all evaluated parameters at 3- and 6-month follow-up periods compared with the baseline ($p < 0.05$). Following IPT, the GCF NO levels significantly decreased in both the treatment groups and remained stable up to 3 months ($p < 0.05$). At 6 months, although there was a trend towards an increase, the reduction of NO levels was still found to be significant in both groups when compared with the baseline ($p < 0.05$) (Table 2).

Table 2 also presents the intergroup comparisons of the evaluated parameters. IPT + Probiotic treatment yielded significantly larger reduction in GCF volume, NO levels, PD and BoP compared with IPT + Placebo at 3- and 6-month follow-up periods ($p < 0.05$).

A relation between periodontal disease and systemic inflammation has been demonstrated in the literature [2, 32–34]. Bacteria and bacterial metabolites and the mediators of inflammation activate iNOS to produce NO [2, 13]. Therefore, by measuring NO and NO_2 and NO_3 levels in biological fluids, the systemic inflammatory response can be also evaluated [14, 16]. Nevertheless, the data concerning the possible effect of periodontal therapy on GCF NO levels are limited [35, 36]. From this standpoint, the present study aimed to evaluate the effects of periodontal therapy

Table 1. Baseline data of the patients in both groups.

	Test group (IPT + probiotic) $n = 18$ Mean ± SD	Control group (IPT + placebo) $n = 18$ Mean ± SD	*p*
Age*	45.4 ± 3.39	46.8 ± 4.25	0.65
Gender† (F/M)	7/11	9/9	0.73
BoP (%)*	84.23 ± 0.7	83.75 ± 1.1	0.693
PD (mm)	4.47 ± 0.61	4.38 ± 0.52	0.766
GCF volume* (μL)	0.56 ± 0.32	0.53 ± 0.14	0.64
NO* (μmol dL^{-1})	86.41 ± 19.18	85.52 ± 22.75	0.89

†*Chi*-square test, *Student's *t* test. Values are statistically significant at $p < 0.05$.
BoP: bleeding on probing; PD: probing depth; GCF: gingival crevicular fluid; NO: nitric oxide.

Table 2. Inter- and intragroup comparisons of the parameters at baseline and 3 and 6 months.

		Baseline Mean ± SD	3 months Mean ± SD	6 months Mean ± SD	p^*
GCF volume (μL)	Test group $n = 18$	0.56 ± 0.32	0.15 ± 0.09	0.11 ± 0.01	0.001
	Control group $n = 18$	0.53 ± 0.14	0.27 ± 0.03	0.19 ± 0.08	0.001
	p^{**}	0.63	0.008	0.002	
NO μmol/dL	Test group $n = 18$	86.41 ± 19.18	39.41 ± 11.83	56.12 ± 15.15	0.001
	Control group $n = 18$	85.52 ± 22.75	53.76 ± 21.02	65.31 ± 19.31	0.001
	p^{**}	0.82	0.001	0.009	
PD (mm)	Test group $n = 18$	4.47 ± 0.61	2.95 ± 0.36	2.92 ± 0.29	0.001
	Control group $n = 18$	4.38 ± 0.52	3.67 ± 0.25	3.8 ± 0.72	0.001
	p^{**}	0.766	0.001	0.001	
BoP (%)	Test group $n = 18$	84.23 ± 2.7	14.6 ± 3.26	12.67 ± 4.98	0.001
	Control group $n = 18$	83.75 ± 1.1	23.11 ± 5.64	19.08 ± 3.55	0.001
	P^{**}	0.693	0.001	0.001	

*One-way ANOVA test,
**Student's t test. Values are statistically significant at $p < 0.05$.
GCF: gingival crevicular fluid; NO: nitric oxide; PD: probing depth; BoP: bleeding on probing.

and the adjunctive usage of probiotic-containing food supplements on the clinical parameters and GCF levels of NO.

When used alone, the clinical measures of periodontitis, such as PD or BoP, feature limitations to provide the clinician with real-time evaluation of the disease status. As GCF passes from the circulation to inflamed periodontal tissues, it carries biological molecules gathered from the neighboring sites. Thus, GCF is an attractive oral fluid due to its ease of collection that enables clinicians to simultaneously sample multiple sites within the oral cavity. The use of GCF suggests a potential diagnostic value to identify periodontal disease activity and responses to therapy [37].

As the NO production is associated with inflammatory diseases, it has been recognised as a marker of inflammation [38]. In different studies, the salivary levels of NO_3, NO_2 and NO of patients with periodontitis have been reported to be higher than those of patients with gingivitis and healthy individuals [30, 39, 40]. The bacterial lipopolysaccharides in the wall of periopathogenic bacteria cause the apoptosis of periodontal ligament through increased iNOS and phosphorylation of C-Jun N-terminal kinase, leading to a localized mircovasculopathy, lasting ischemia and consequent permanent damage to endothelial and surrounding periodontal tissues [41]. On the other hand, the effect of periodontal treatment on salivary NO levels remains controversial [10, 27, 30]. The whole saliva is a complex mixture composed of blood, GCF, food debris, bacteria and their products. Therefore, the NO levels in saliva may be affected by various factors [29]. Different results obtained from the studies may be attributed to the degradation of salivary NO by various salivary factors [27]. Furthermore, smoking, saliva collection methodology and salivary proteins may influence the measurement of NO metabolites [10, 30].

Regarding the results of the present study, a significant reduction in the GCF NO levels has been demonstrated after the IPT. Thus, the usage of probiotic tablets as an adjunct in the IPT achieved significantly more reduction of the GCF NO levels in favour of the test group in both the 3- and 6-month follow-up. NO may both directly and indirectly play a role in the progression of periodontal diseases by modulating the pro-inflammatory cytokine production [38]. In the pristine periodontium, NO production is physiologically secreted by cNOS, mostly from endothelial cells that maintain the vascular integrity. With the progression of periodontal disease, NO synthesis is surmised to be produced by iNOS, which is responsible for the maintenance of the inflammation [42]. Therefore, the findings of the present study agree with those of the other studies suggesting an increased NO production due to the induction of iNOS in periodontally diseased tissues [28, 42, 43].

The effect mechanisms by which probiotics modulate immunity in various parts of the body have been studied. Among these parts, the oral cavity specifically is of major importance. The enhancement of local immune responses along with systemic immunity by probiotics may present new opportunities in preventing infections at peripheral mucosal surfaces, such as in the respiratory and urogenital tract structures [21]. However, the literature lacks data to explain the molecular mechanisms for the adjunctive beneficial effect of probiotic supplementation in the field of periodontology. The mechanisms that are possibly responsible for the beneficial effects of probiotics include the interaction with pathogens and production of the antimicrobial substances and the modulation of the host immune response [26, 44]. The observed effect may be due to the host immune-response modulation by inhibiting the NO production of iNOS.

As previously mentioned, the NO levels may be used as markers of inflammation, disease severity and pathogenesis, requiring reliable and sensitive analytical techniques for quantitation of NO production and iNOS activity [31]. NO analysis can be performed by different direct and indirect methods, including gas and liquid chromatography, electron paramagnetic resonance and mass spectrometry. However, disadvantages, such as short half-life, low *in vivo* concentrations of NO, unsuitableness for the clinical settings due to instrumentation requirements and inexpedience in processing large numbers of samples, reduce the implementation of these methods for evaluation of biological samples [31, 45]. To overcome these challenges, stable metabolites of NO are measured, such as NO_2 and NO_3, with NO_2 as the only stable end-product of the autoxidation [31].

In the present study NO_2 and NO_3 concentrations were determined in GCF through the reduction of NO_3 by vanadium (III) and detection with Griess reagents. Significant reductions were observed in the total NO levels after the IPT, and the adjunctive usage of probiotic tablets yielded significantly more reduction in the GCF NO levels. Based on these results, the GCF NO may be suggested as a useful marker for periodontal inflammation and disease pathogenesis. The composition of saliva is affected by various factors, such as systemic diseases and salivary glands and the periodontal tissues and caries, GCF may possess high specificity to reflect the periodontal status, as its biomarkers are mainly secreted from the surrounding individual periodontal tissues. However, Poorsattar et al. [41] suggested the salivary total NO, NO_2 and NO_3 to be more sensitive biomarkers than the total NO, NO_2 and NO_3 content of GCF. In the same study, the ROC curves demonstrated that the saliva was more reliable in the differentiation of periodontitis from gingivitis and healthy periodontium. However, different from the present study design, their study included a healthy control group, and the NO levels were analysed by enzyme-linked immunosorbent assay.

The limitation of the present study may be its small cohort size due to difficulties in the selection of patients and follow-up. Larger-sample-size studies including patients with different periodontal conditions are required to define the role of probiotics and GCF NO levels in the management of periodontal diseases.

Conclusions

According to the results of the present study, the GCF NO content may feature a potential use as a marker of inflammation in periodontal diseases. In addition, *L. reuteri*-containing probiotic food supplements can be suggested as an adjunct to the IPT. However, further studies are needed to clarify the underlying mechanisms of probiotic action.

Notes

1. ProDentis®, BioGaia, Stocholm, Sweden.
2. Periopaper®, Oraflow, New York, USA.
3. Periotron 8000®, Oraflow, New York, USA.
4. PCPUNC15, Hu Friedy, Chicago, USA.

Disclosure

No potential conflict of interest was reported by the authors.

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