J Clin Periodontol 2016; doi: 10.1111/jcpe.12542

Journal of Clinical Periodontology

Stimulation of the nitrate-nitrite-NO-metabolism by repeated lettuce juice consumption decreases gingival inflammation in periodontal recall patients: a randomized, double-blinded, placebo-controlled clinical trial

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Jockel-Schneider Y, Goßner SK, Petersen N, Stölzel P, Hägele F, Schweiggert RM, Haubitz I, Eigenthaler M, Carle R, Schlagenhauf U. Stimulation of the nitrate-nitrite-NO-metabolism by repeated lettuce juice consumption decreases gingival inflammation in periodontal recall patients: a randomized, double-blinded, placebo-controlled clinical trial. J Clin Periodontol 2016; doi: 10.1111/jcpe.12542.

Abstract

Aim: This prospective, parallel group, two-armed, double-blind, placebo-controlled randomized trial evaluated the impact of dietary nitrate consumption on gingival inflammation in periodontal recall patients.

Material and Methods: Forty-four (23 test/21 placebo) periodontal recall patients with chronic gingivitis were enrolled. At baseline, gingival index (GI), plaque control record (PCR) and salivary nitrate level (SNL) were recorded, followed by sub- and supragingival debridement. Subsequently, participants were randomly provided with 100 ml bottles of a lettuce juice beverage to be consumed 3× daily over 14 days, containing either a standardized amount of nitrate resulting in an intake of approximately 200 mg nitrate per day (test) or being devoid of nitrate (placebo).

Results: At baseline, mean GI, PCR and SNL did not differ significantly between the groups. At day 14, mean GI of the test group was significantly reduced compared to baseline and significantly lower ($p = 0.002$) than in the placebo group (GI 0.3 *versus* 0.5). Also, mean SNL in the test group was significantly higher than in the placebo group (54.0 μg/ml *versus* 27.8 μg/ml; $p < 0.035$). Mean PCR did not change significantly in both groups.

Conclusions: Dietary nitrate consumption may be a useful adjunct in the control of chronic gingivitis.

Conflict of interest and source of funding statement
The authors declare that there are no conflicts of interest.
This study was partially funded by a grant of the Commission for Young Scientists and Gender Equality, University of Wuerzburg (2.2-220.522-97/15_1).

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Key words: gingivitis; periodontal disease; periodontitis

Accepted for publication 6 March 2016

In industrialized countries, chronic gingivitis belongs to the most prevalent oral diseases affecting, e.g. more than 90% of the German population in varying severity (Holtfreter et al. 2010). The onset of gingival inflammation is accompanied by the establishment of bacterial dysbiosis, i.e. a significant shift in the composition of polymicrobial oral biofilms (Hajishengallis et al. 2012) towards the overgrowth of specific, proteolytic and mostly Gram-negative bacterial species (Hajishengallis et al. 2012). Data from a clinical study evaluating the oral health of individuals living under stone age conditions without proper individual plaque control suggest that nutrition pattern may considerably interfere with the clinical expression of gingivitis (Baumgartner et al. 2009). In numerous studies, the ingestion of dietary nitrate has been proven to exert many beneficial and clinically relevant effects on the general health. Among them are the maintenance of vascular homeostasis and the reduction of platelet aggregation and chronic inflammation in general. All these effects are based on the conversion of nitrate to various reactive nitrogen intermediates (RNIs) such as nitric oxide (NO), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) *via* nitrite. NO affects the release of numerous inflammatory mediators (Weitzberg & Lundberg 2013). A marked decrease of bioavailable NO released by the endothelial NO synthase (eNOS) is one of the major sequelae of the metabolic syndrome. In an animal study, it has been demonstrated that the nutritional supplementation of nitrate in eNOS-deficient mice was able to reverse various metabolic syndrome associated pro-inflammatory vascular symptoms (Carlstrom et al. 2010). As the gingival sulcus shares many immunological and structural traits with the gut and has to resist a comparable microbial load, the influence of NO-mediated variations in the microcirculatory perfusion and its consequences for the inflammatory status of the gingival tissues is of major clinical interest.

This study evaluated the influence of the repeated consumption of a nitrate-containing lettuce juice on the clinical signs of chronic gingivitis in a cohort of periodontal recall patients.

Material and Methods

Experimental lettuce juice beverage

The experimental lettuce juices were prepared from a commercially available lettuce (*Lactuca sativa* L.) juice concentrate (8°Brix, lot-number CH 50005720/001; Ernteband Fruchtsaft, Winnenden, Germany) at the Institute of Food Technology, Section Plant Foodstuff Technology and Analysis of the University of Hohenheim. In order to ensure standardized nitrate levels in the test juice, it was firstly depleted of its genuine nitrate content by ion exchange chromatography, and subsequently adjusted to 667 mg/l by the addition of food-grade potassium nitrate (Merck, Darmstadt, Germany), thus mimicking the genuine concentrations in lettuce. The nitrate-depleted lettuce juice served as placebo. To improve patient acceptance, both the placebo and the test lettuce juice were flavoured by an identical allergen-free chamomile-honey flavour (Aromen4you, Osnabrueck, Germany), sucralose and malic acid.

Study design

This investigation was designed as a prospective, parallel group, two arm, double-blind, placebo-controlled randomized clinical trial. The study protocol was prepared in accordance with the declaration of Helsinki and met GCP criteria; it was approved by the ethics committee of the University of Wuerzburg (file# 211/14). All subjects included had signed a written informed consent prior to their inclusion.

Study population

Study patients were recruited between August and November 2014 from periodontal recall patients visiting the Department of Periodontology of the University Hospital of Wuerzburg for routine periodontal supportive care being performed typically in 3–6 months intervals with annual re-evaluations of their periodontal status.

Blinding and randomization

Patients were allocated using a computer-generated randomization list in blocks of six either to the test or the placebo group. Assignment to a participant number was done according to the chronological order of enrolment in the study. All therapeutic and diagnostic interventions were performed at the Department of Periodontology of the University Hospital of Wuerzburg. All examinations were performed by two experienced, inter- and intra-calibrated periodontists not being involved in patient assignment and hand out of the experimental juices respectively. Thus, following a double-blind design, neither the periodontists performing the measurements and supportive periodontal therapy nor the study patients themselves were aware of the assignment to the test or placebo group respectively. A code break for the randomization was kept in a sealed envelope at the Department of Periodontology.

Eligibility criteria were a history of treated periodontal disease with subsequent inclusion in regular (2–4× annually) periodontal supportive care, the presence of mild to moderate chronic gingivitis as defined by a gingival index score of GI 1 or GI 2 at a minimum of three teeth and total of at least 10 natural teeth *in situ*.

Exclusion criteria were the manifestation of severe gingivitis (GI > 2) at any tooth, age <18 years, pregnancy or breastfeeding, manifestation of inflammatory oral mucosal diseases other than gingivitis, xerostomia (salivary flow <0.1 ml/min), inability to perform regular oral home care, known allergies and intolerances to any of the ingredients of the test juices as well as intellectual inability to comprehend and comply with the aims of the study.

Screening and recruitment

After comprehensive face to face discussion with a staff member about the risks and benefits of study participation ultimately 44 subjects, age 46–77 years (16 male/28 female) gave their written informed consent, and were subsequently enrolled in the trial.

Baseline examination and supportive periodontal therapy

At baseline, the following parameters were recorded:

Gingival index

The extent of gingival inflammation was assessed visually on the buccal aspect of all teeth using the Lobene modification of the gingival index (Loe et al. 1965, Lobene et al. 1986).

Plaque control record (PCR)

The extent of plaque coverage on the mesial, distal, buccal and oral aspects of all teeth was documented using the plaque control record (O'Leary et al. 1972).

Salivary nitrate level (SNL)

For the assessment of salivary nitrate levels, stimulated saliva was collected by chewing on paraffin wax. Aliquots of 3.0 ml saliva were subsequently stored in Eppendorf vials at −25°C until further analysis.

Periodontal and general health profile

Additionally, a periodontal and general health profile of the study patients was assessed comprising the number of natural teeth, probing pocket depth, bleeding on probing as well as age, body mass index, smoking habits and prosthodontic status.

Subsequently, all study patients received standardized supportive periodontal therapy comprising professional supra- and subgingival debridement but without any oral hygiene instructions.

Immediately afterwards the subjects were randomly assigned to one of the two experimental groups (test, placebo) and provided with 42 bottles, each containing 100 ml of the assigned lettuce juice to be consumed three times daily over the time course of 14 days. Consumption of 300 ml of the nitrate-containing test juice resulted in a daily intake of 200 mg nitrate being close to the acceptable daily intake for nitrate of 3.7 mg/kg or 222 mg for a 60 kg person as set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Participants were instructed to keep a consumption diary to monitor the time of consumption and were asked to return all supplied bottles to further verify the compliance with the study protocol. Furthermore, in order to minimize the uptake of dietary nitrate from other sources, all participants were provided with an instruction leaflet listing an array of nitrate-rich foods, which were not allowed to be consumed during the study period (see Appendix S1). As vegetables contribute approximately up to 70% of the total dietary nitrate intake (Ysart et al. 1999), subjects were instructed not to eat, e.g. nitrate-rich leafy or root vegetables and cabbages. They were also asked to limit their potato consumption and avoid cured meat products for the duration of the clinical trial. Assuming that the average dietary nitrate uptake of an adult is 93 mg/day (Ysart et al. 1999), compliant study participants should have limited their dietary nitrate intake from other sources thus to a maximum of 28 mg/day. All study patients were Caucasians and residents of the city of Wuerzburg or its surroundings. Their habitual nutrition pattern reflected the local diet rich in potatoes and meat.

Subjects were also instructed to refrain from applying antiseptic mouthwash for the duration of the study, as these are known to suppress oral nitrate-reducing bacteria, thereby significantly interfering with the physiological effects of nitrate consumption (Petersson et al. 2009).

Re-evaluation (day 14 of the study)

After 14 days of juice consumption, the study patients were re-evaluated and saliva samples for SNL analysis were taken again, followed by the assessment of GI and PCR scores.

Analysis of salivary nitrate levels

Salivary nitrate level analysis was performed at the laboratory of the Chair for Plant Foodstuff Technology of the Institute of Food Science and Biotechnology at the University of Hohenheim. Salivary samples were suspended in ultrapure water and proteins and other impurities affecting the analysis were precipitated by Carrez clarification. After subsequent centrifugation and membrane filtration, SNL was determined by High-Performance Anion-Exchange Chromatography with Suppressed Conductivity Detection (HPAEC-CD).

Statistical analysis

Group size calculation assumed an observed mean GI score of GI = 0.9 and SD for GI of ± 0.5. To verify a clinically meaningful difference in the observed mean values of 50% between test and placebo group at the end of the study with a power of 0.8, a sample size of 2×20 study patients has been calculated.

A Chi-square test with maximum likelihood method was performed to analyse differences between categorical variables. As normal distribution for the recorded data could not be assumed, Mann–Whitney *U*-test was used for the analysis of independent samples, and the Wilcoxon signed-rank test for the analysis of paired samples. The level of significance was set to $p \leq 0.05$.

All statistical analyses were performed by a professional statistician using the WinMEDAS statistical software package (pdv software, Goslar, Germany).

Primary research question

Does the repeated consumption of a nitrate-containing lettuce juice over a time course of 14 days after supportive periodontal care significantly reduce the clinical signs of gingivitis as recorded by the gingival index (Loe et al. 1965, Lobene et al. 1986) in a cohort of periodontal recall patients when compared to a cohort of periodontal recall patients receiving identical periodontal supportive care and consuming an identical but nitrate-depleted lettuce juice?

Results

Assignment and compliance

Out of a total of 44 enrolled study patients (23 test/21 placebo), five individuals dropped out during the course of the trial on private reasons. Three of them had been assigned to the test and two to the placebo group.

Periodontal and general health profile

Periodontal and general health characteristics of the study population are depicted in Table 1.

There were no significant differences between the two groups in terms of age, smoking, body mass index, number of teeth, severity of periodontal disease or bleeding on probing. With the exception of one

subject displaying a removable partial denture, all other study participants were wearing no or fixed prosthodontics.

Gingival index, plaque control record, salivary nitrate level

The results of the analysis of the recorded GI, PCR and SNL values are depicted in Table 2.

Gingival index

At baseline, recorded GI mean scores did not differ significantly between the groups (GI test group: 0.6 ± 0.3 SD; GI placebo group: 0.6 ± 0.4 SD). Fourteen days later at re-evaluation, the observed mean GI score of the test group (GI: 0.3 ± 0.2) was significantly reduced compared to baseline ($p = 0.00015$) and significantly ($p = 0.002$) lower than in the placebo group (GI: 0.5 ± 0.2).

The frequency distribution of the relative numbers of teeth displaying GI categories 0, 1 or 2 in each patient is shown in Fig. 1. In the placebo group, the distribution of the different GI scores displayed only minor, insignificant variation between baseline and day 14. Meanwhile in the test group, a significant decrease in the gingivitis-associated categories GI 1 and GI 2 occurred with a concomitant significant increase in clinically uninflamed sites (GI = 0). Within the observation period, overall 95% of the dietary nitrate group patients exhibited a reduction in the observed mean GI values by contrast to only 55% of the placebo group patients.

Plaque control record

The results of the PCR analysis are shown in Table 2. Mean PCR scores recorded at baseline and at day 14 did neither differ significantly between nor within the experimental groups.

Salivary nitrate level

The results of the SNL analysis are depicted in Table 3. At baseline, mean SNL of the test group (35.5 μg/ml ± 29.6) did not differ significantly from mean SNL recorded for the placebo group (51.9 μg/ml ± 52.2). After 14 days of lettuce juice consumption, mean SNL of the test group (54.0 μg/ml ± 59.2) was significantly higher ($p = 0.035$) than mean SNL of the placebo group (27.8 μg/ml ± 37.7).

Discussion

This clinical intervention trial demonstrated an attenuating effect of dietary nitrate on gingival inflammation. This is in line with the findings of other studies and systematic reviews evaluating the impact of nutrition on the aetiology and progression of chronic diseases like atherosclerosis, diabetes, caries, gingivitis and periodontitis (Baumgartner et al. 2009, Hujoel 2009, Dias et al. 2015).

Dietary nitrate, mainly originating from vegetables (Ysart et al. 1999) is absorbed very effectively in the stomach and the upper part of the small intestine. Approximately 25%of the circulating nitrate is actively accumulated in the salivary glands (Spiegelhalder et al. 1976).

Although dietary nitrate has a bioavailability of about 90–95% in humans (Kortboyer et al. 1997), huge intra- and inter-individual differences in the metabolism of dietary nitrate are to be expected (Tannenbaum et al., 1976). The wide range of individually recorded salivary nitrate

Table 1. Periodontal and general health profile

	Dietary nitrate (test) group ($n = 20$)	Placebo group ($n = 19$)	p*
Age (years)	59.0 ± 9.2	62.4 ± 8.0	n.s.
BMI (kg/m^2)	25.9 ± 3.3	27.3 ± 6.8	n.s.
Male (%)	43	56	n.s.
Non-smoker	20	16	n.s.
No. of teeth	25.7 ± 2.2	24.2 ± 4.9	n.s
No. of teeth with pocket depth ≤3.5 mm	15.0 ± 7.4	11.8 ± 7.1	n.s
No. of teeth with pocket depth >3.5 mm ≤5.5 mm	10.1 ± 6.2	11.5 ± 4.8	n.s
No. of teeth with pocket depth >5.5 mm	0.7 ± 1.3	0.8 ± 1.9	n.s
Bleeding on Probing BoP per % all teeth tooth in %	11.6 ± 14.8	16.0 ± 9.8	n.s

n.s. = not significant.
*p from and Fisher und Yates test for gender and smoking, all others Mann–Whitney U-test.

Table 2. Gingival index (GI) and plaque control record (PCR) at baseline and at day 14 (re-evaluation)

	Dietary nitrate group ($n = 20$)					Placebo group ($n = 19$)					
	Mean	SD	Median	68% confidence interval of median		Mean	SD	Median	68% confidence interval of median		p*
GI baseline	0.6	0.2	0.5	0.5	0.6	0.6	0.4	0.4	0.4	0.5	0.15
GI day 14	0.3	0.1	0.2	0.2	0.3	0.5	0.2	0.4	0.4	0.5	0.002
PCR [%], baseline	33.0	13.3	30.5	26.7	35.9	39.0	20.2	31.9	29.0	43.4	0.41
PCR [%], day 14	30.7	14.9	27.2	23.0	32.7	40.5	19.7	36.1	32.8	45.2	0.079

*p from Mann–Whitney U-test.

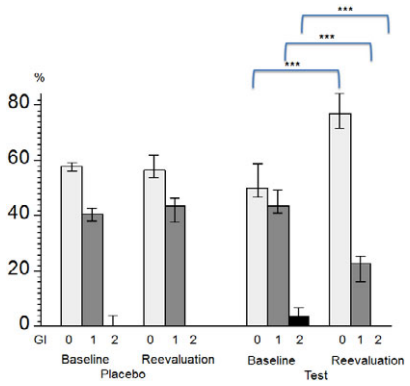

Fig. 1. Frequency distribution of the percentage of teeth displaying gingival index categories 0, 1 or 2 in each patient (median values) ***$p < 0.001$ (Wilcoxon exact test).

Table 3. Salivary nitrate level (SNL)

	Dietary nitrate group $n = 20$		Placebo group $n = 19$		p*
	Mean	SD	Mean	SD	
SNL baseline (μg/ml)	35.5	29.6	51.9	52.2	0.61
SNL day 14 (μg/ml)	54.0	59.2	27.8	37.7	0.035

*p from Mann–Whitney U-test.

concentrations in this study (e.g. at baseline: min: 0 μg/ml, max: 176.9 μg/ml) and the high standard deviations clearly confirm these findings.

Anaerobic bacteria residing on the tongue reduce salivary nitrate to nitrite (Duncan et al. 1995, Li et al. 1997). Under acidic and reducing conditions, nitrite is rapidly protonated to form nitrous acid (HNO_2), which further decomposes to NO and other RNIs such as nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) (Lundberg et al. 2004, Weitzberg & Lundberg 2013). Next to its vasodilatory, blood-pressure-lowering properties, NO exerts several other potentially beneficial effects in humans, including inhibition of platelet aggregation and inflammatory cell recruitment, preservation of endothelial function and improvement of mitochondrial efficiency (Lundberg et al. 2008, Webb et al. 2008, Larsen et al. 2010).

Reactive nitrogen intermediates also display antimicrobial properties against numerous pathogens including, bacteria, fungi, parasites, viruses and even inhibitory effects against tumour cells (De Groote & Fang 1995, Jenkins et al. 1995). Furthermore, NO suppresses T-cell proliferation and activity, the synthesis of interleukin-6, prostaglandin E_2 and thromboxane in macrophages, as well as the release of platelet activating factor and histamine from mast cells (Kubes et al. 1991).

As this trial primarily focused on the clinical impact of the ingestion of dietary nitrate, it may only be speculated which of the aforementioned mechanisms and pathways contributed to the present findings. To the best of our knowledge, a clinical trial on the interference between dietary nitrate intake and gingival inflammation has not been published before.

The minor improvement of gingival inflammation observed in the placebo group despite professional periodontal supportive care may not only be explained by the intentional lack of any supportive oral hygiene instructions during the course of the trial, but may also reflect that established chronic gingivitis is a disease entity markedly different from experimentally induced gingivitis (Deinzer et al. 2007) and most likely less susceptible to mechanical plaque control.

In conclusion, our findings suggest that the ingestion of dietary nitrate may be a clinically useful adjunct in the control of chronic gingivitis in periodontal recall patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Dietary instructions for the duration of the clinical trial.

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Clinical Relevance

Scientific rationale for the study: This double-blinded prospective study evaluated the influence of dietary nitrate consumption on chronic gingivitis in periodontal recall patients.

Practical implications: Stimulation of the nitrate–nitrite–NO metabolism by repeated lettuce juice uptake significantly reduced gingival inflammation independent of the level of plaque control.

Principal findings: The regular uptake of dietary nitrate may have the potential to become an inexpensive, easily implementable and effective therapeutic – possibly even prophylactic – complement in the control of chronic gingivitis.