Brief Communication

Total salivary nitrates and nitrites in oral health and periodontal disease

Gabriel A. Sánchez b,⇑, Valeria A. Miozza a, Alejandra Delgado a, Lucila Busch a

a Biophysics Unit, School of Dentistry, University of Buenos Aires, Marcelo T de Alvear 2142 (1122AAH), Buenos Aires, Argentina
b Pharmacology Unit, School of Dentistry, University of Buenos Aires, Marcelo T de Alvear 2142 (1122AAH), Buenos Aires, Argentina

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A B S T R A C T
It is well known that nitrates are increased in saliva from patients with periodontal disease. In the oral cavity, nitrates may derive partly from the reduction of nitrates by oral bacteria. Nitrates have been reported as a defence-related mechanism. Thus, the aim of the present study was to determine the salivary levels of total nitrate and nitrite and their relationship, in unstimulated and stimulated saliva from periodontal healthy subjects, and from patients with chronic periodontal disease. Nitrates and nitrites were determined in saliva from thirty healthy subjects and forty-four patients with periodontal disease. A significant increase in salivary nitrates and nitrites was observed. Nitrates and nitrites concentration was related to clinical attachment level (CAL). A positive and significant Pearson’s correlation was found between salivary total nitrates and nitrites. Periodontal treatment induced clinical improvement and decreased nitrates and nitrites. It is concluded that salivary nitrates and nitrites increase, in patients with periodontal disease, could be related to defence mechanisms. The possibility that the salivary glands respond to oral infectious diseases by increasing nitrate secretion should be explored further.

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Introduction

Nitrate is found in high concentrations in vegetables, especially leafy ones, such as lettuce and spinach. In human, ingested nitrate is absorbed from the duodenum and upper ileum into the blood stream and concentrated in the salivary glands by an active transport system [1], increasing concentrations up to 10 times compared to plasma [2]. About 25% of the nitrate in plasma is secreted into the oral cavity as a component of saliva. Nitrate-reducing bacteria in the human oral cavity can reduce salivary nitrate to nitrite [3], which is further reduced to nitric oxide (NO) by nitrite reducing bacteria [4].

Although nitrate has historically been associated with deleterious effects in humans, such as infant methaemoglobinaemia [5], new evidence has suggested a beneficial, antimicrobial role for inorganic nitrate in several systems in humans, including the gastrointestinal tract, oral cavity and skin [6,7]. In relation to the oral cavity, patients with a high concentration of nitrate in saliva and an oral flora with a high capacity to reduce nitrate to nitrite, have significantly less caries history than those with low amounts of nitrate in saliva and an oral flora with low capacity to reduce nitrate to nitrite [8]. Besides, salivary nitrate and nitrites are increased in patients with oral candidiasis [9] and stress promotes salivary nitrate secretion and nitrite formation, which may play important roles in gastric protection against stress-induced injury via the nitrate-dependent NO pathway [10].

Nitrite concentration is increased in unstimulated and stimulated saliva of patients with periodontal disease [11,12]. The activity and expression of the inducible isoform of the enzyme nitric oxide synthase (NOS), is increased in gingival tissue of patients with gingivitis or chronic periodontitis [13,14], inducing an increase in NO production. NO, a highly reactive free radical gas, decaes into equal amounts of nitrite and nitrate in aqueous solutions [15]. In addition, NO production from nitrite in gingival tissue is pH dependent, being enhanced at pH below 5 [16]. On the other hand, Aurer et al. [17] found a decrease in nitrite production in unstimulated saliva of patients with periodontitis.

Changes in total salivary nitrate concentration and its relation with nitrite concentration in periodontal disease have not been described. Hence, the purpose of this study was to determine the levels of total nitrate and nitrite, in unstimulated and stimulated saliva from periodontally healthy subjects, and from patients with chronic periodontal disease. The influence of appropriate periodontal treatment on salivary total nitrate and nitrite concentration was also evaluated.

Materials and methods

Subjects

A total of 74 adult subjects, 44 patients with chronic periodontitis (31 males and 13 females) and 30 periodontally healthy subjects...
(20 males and 10 females) participated in this study. They were enrolled at a private dental clinic and consented to participate. Before the study, a dietary and medical history of the subjects was ascertained and all received a complete dental check-up. General exclusion criteria included smokers, diabetes, immunosuppression, vegetarians and medicine use. Local exclusion criteria included: oral infections other than periodontal disease and bad hygiene habits. Chronic periodontitis was diagnosed by measuring the clinical attachment level loss (CAL) and probing pocket depth (PPD) using a calibrated manual probe. Inclusion criteria included participants with ≥2 interproximal sites with CAL ≥ 4 mm and ≥2 interproximal sites with PPD ≥ 5, who had not previously received periodontal treatment.

The protocol was approved by the Ethics Committee of the School of Dentistry, University of Buenos Aires, and the study was conducted in accordance with the Declaration of Helsinki (version 2008).

Periodontal treatment

After periodontal diagnosis, subjects were given thorough oral hygiene instruction including use of a toothbrush, wooden sticks and interdental brushes. Thereafter, only patients with periodontal disease received 2–5 one-hour session of thorough supra-gingival scaling, sub-gingival scaling and root planning of all tooth surfaces under local anaesthesia [18]. Three months later an evaluation of treatment was carried out and all clinical parameters were assessed again as described above.

Collection of saliva

Individuals were encouraged to follow their normal daily nutritional regimen. No specific instructions regarding food or fluid intake were given [17]. For collection of saliva subjects were advised to have 3 h fasting [12]. Saliva was collected under both stimulated (obtained after chewing paraffin for 5 min) and unstimulated conditions by spitting into an ice-cooled graduated vessel. Subjects spat out every 30 s for 5 min. The volume of saliva was recorded and expressed as ml/min. The resulting saliva was stored in aliquots at −20 °C until determinations were performed. Saliva was collected at 10 am by one calibrated examiner the day after the initial diagnosis and the day after the post-treatment periodontal evaluation.

Determination of nitrate and nitrite

Nitrate and nitrite levels were measured, in unstimulated and stimulated saliva, using the Griess colorimetric reaction [19]. Griess reagent is a 1:1 mixture of 1% sulphanilamide and 0.1% N-naphthylethylenediamine dichloride in 5% orthophosphoric acid (v/v). This reagent reacts with nitrite and produces a purple azo dye endo-product, which can be measured spectrophotometrically with a maximum absorbance at 570 nM. Duplicate samples of saliva (20 μl) diluted 1:100 and 1:200 were transferred to a 96-well ELISA plate (Cayman Chemical Co., Ann Arbor, MI, USA). The method for measurement of total nitrate/nitrite concentration involves a simple two-step process. The first step is the conversion of nitrate to nitrite utilising nitrite reductase, being NADPH its essential cofactor. The interference of NADPH with the chemistry of the Griess reagents is avoided by using small amounts of NADPH in conjunction with a catalytic system for recycling spent NADP⁺ back to NADPR. The second step is the addition of the Griess Reagents which convert nitrite into the deep purple azo compound in approximately 10 min. Photometric measurement of the absorbance accurately determines nitrite concentration. In this study, nitrite (avoiding the nitrate reductase) and total nitrate/nitrite concentration was determined. In order to obtain calibration curves, duplicate samples of sodium nitrate at concentrations of 2.5, 5, 10, 15, 20, 25, 30, 35 and 40 μM were also included in the plate.

Statistical analysis

Statistical significance of differences was determined by analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test. Multiple linear regression analysis was used to establish the relation of total nitrates and nitrites with age, clinical parameters and saliva conditions. In order to compare salivary nitrate/nitrite between the initial visit and after periodontal treatment, Student’s paired t-test was used. Pearson’s correlation analysis was used to evaluate the relation between total nitrates and nitrites. Data analysis was performed using GRAPHPAD Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). The level of statistical significance is given when p < 0.05.

Results and discussion

In this study it was observed that total nitrate and nitrite concentration was significantly increased in unstimulated and stimulated saliva from patients with periodontal disease (Fig. 1).

It is known that nitrates from plasma, which derive from diet, are concentrated in salivary glands and secreted into the oral cavity [1]. Thus, the ingestion of sodium nitrate [20] greatly increases salivary levels of nitrate and nitrite. Conversely, enriched or low nitrate diets do not influence salivary nitrite and nitrate levels [17]. Since in this study patients followed a normal daily nutritional regimen, the increment of nitrates observed could be hardly ascribed to differences in patients’ diet. Although the plasma nitrate and nitrite levels in the subjects were not determined and therefore it is not possible to disassociate the oral and blood-recycling pathway, the fact that nitrate and nitrite levels decreased after periodontal treatment points to a relation with the disease. Dietary nitrate is the basis for a non-immune defence mechanism against oral and intestinal pathogens in humans and animals [2]. Nitrite, derived from nitrate through nitrate reducing bacteria on the tongue surface, is considered cytocidal and cytostatic to common oral pathogens involved in caries [8,21,22] and in periodontal disease [23]. Therefore, an increase in nitrate secretion and a subsequent increase in salivary nitrite may contribute to the overall protective effect against those infections conditions, affecting both hard and soft oral tissues. It is known that salivary glands may respond to periodontitis by enhancing the protective potential of saliva [24]. Thus, it is likely that the increment in salivary nitrate–nitrite concentration, in patients with periodontal disease, was due to an increase in nitrate secretion as a response of salivary glands to the inflammatory process. In concordance with this hypothesis, it has been reported that patients with oral candidiasis have increased salivary nitrates and nitrites concentration [9].

A multiple linear regression analysis showed a relevant relation between the salivary concentrations of total nitrates and nitrites with CAL in both unstimulated (Table 1) and stimulated saliva (Table 2). This result shows that total salivary nitrates and nitrites increased concomitantly with CAL and supports the above hypothesis.

Salivary nitrate showed a positively significant relation with pH. Nitrate reduction, in human oral cavity, takes place in the dorsal surface of the tongue that represents an ecological niche for anaerobic bacteria [3]. In this regard, it was reported that anaerobic incubation of saliva containing a mixture of oral bacteria, in the presence of nitrate/nitrite substrate and glucose, resulted in a higher pH than was found in controls in the absence of nitrate/
These results suggest that the presence of these electron acceptors repressed acid fermentation, or increased alkali production, or consumed acid produced, thus reducing salivary acidity. This finding identifies salivary nitrate as a possible ecological factor in reducing oral acidity [25] and could explain the positive relation found between nitrate and pH in this study. Because salivary pH of subjects ranged between 6.7 and 7, and for conversion of nitrite to nitric oxide pH about 5–6 is needed [16,23], pH hardly could be a factor in nitrite levels.

In this study, Pearson’s correlation analysis showed significant positive correlation between total nitrates and nitrites in unstimulated and stimulated saliva (Fig. 2). Nitrates in the oral cavity derived from salivary glands but no nitrite was detected in parotid saliva [26]. Salivary nitrite may derive from nitrate or from NO, which direct measurements from body fluids is hard to perform [17]. In healthy oral cavity, the origin of nitrite may be the oxidation of NO, derived from nerves fibres in salivary glands [27] or be produced by nitrate-reducing bacteria [8]. In the micro-environment of periodontal disease, the presence of inflammatory stimuli capable of inducing the NO production by the inflammatory cells is already well established [14]. Thus, in the oral cavity of healthy subjects nitrite was formed, and its concentration was directly related to salivary nitrate. During periodontal disease this process was increased, resulting in an increment in salivary nitrate and nitrite concentration. This fact explains the good correlation between total salivary nitrates and nitrites. However, it cannot be discharged other origin for nitrites in periodontal patients, such as the oxidation of NO produced by the inflammatory cells. But this would be the responsible of nitrite increase in crevicular fluid rather than in whole saliva, where its contribution could be negligible. On the other hand, it was postulated that, under certain conditions, nitrite concentration could increase in saliva caused by an increase in the population of nitrate-reducing bacteria [28].

Periodontal treatment attenuates salivary nitrate and nitrite concentration in patients group (Fig. 3, upper panel). Because in healthy subjects a re-assessment after 3 months did not show differences with respect to previous values (Fig. 3, lower panel), this result is in concordance with the above hypothesis, that there is a relation between salivary nitrate and nitrite concentration with periodontal status. The relationship between NO levels in saliva and the severity of periodontitis has been described [11]. In the present study, it is reported the relation not only of nitrites but also of nitrates, with periodontal status. The relevance of this study is the new concept about salivary nitrite origin, in patients with periodontal disease. In general, nitrite increase was related to the inflammatory process, but these results show that it is derived

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**Table 1**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Salivary [total nitrate]</th>
<th>Salivary [nitrite]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.05 (p = 0.38)</td>
<td>0.082 (p = 0.38)</td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td>0.19 (p = 0.015)</td>
<td>−0.118 (p = 0.147)</td>
</tr>
<tr>
<td>pH</td>
<td>0.13 (p = 0.068)</td>
<td>0.10 (p = 0.15)</td>
</tr>
<tr>
<td>CAL (mm)</td>
<td>0.793 (p &lt; 0.0001)</td>
<td>0.8 (p &lt; 0.0001)</td>
</tr>
<tr>
<td>PPD (mm)</td>
<td>0.095 (p = 0.448)</td>
<td>−0.14 (p = 0.26)</td>
</tr>
</tbody>
</table>

Data are β values and, in parenthesis, is shown the significance expressed as p value.
CAL, clinical attachment level; PPD, probing pocket depth.

**Table 2**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Salivary [total nitrate]</th>
<th>Salivary [nitrite]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.07 (p = 0.44)</td>
<td>0.001 (p = 0.99)</td>
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<td>Flow rate (ml/min)</td>
<td>−0.015 (p = 0.86)</td>
<td>0.073 (p = 0.38)</td>
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<tr>
<td>pH</td>
<td>0.16 (p = 0.022)</td>
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<tr>
<td>CAL (mm)</td>
<td>0.59 (p &lt; 0.0001)</td>
<td>0.58 (p = 0.007)</td>
</tr>
<tr>
<td>PPD (mm)</td>
<td>0.23 (p = 0.049)</td>
<td>0.027 (p = 0.87)</td>
</tr>
</tbody>
</table>

Data are β values and, in parenthesis, is shown the significance expressed as p value.
CAL, clinical attachment level; PPD, probing pocket depth.

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**Fig. 1.** Salivary concentration of total nitrates (●) and nitrites (□) in unstimulated and stimulated saliva from periodontal healthy subjects and from patients with chronic periodontitis. Bars represent mean ± SEM. ***Significantly different from healthy values (p < 0.001).

**Fig. 2.** Pearson’s correlation analysis between salivary concentration of total nitrates and nitrites in unstimulated and stimulated saliva from the all subjects studied.
although further studies are needed to confirm this hypothesis.

Fig. 3. Upper panel: salivary concentration of total nitrates and nitrites before (■) and after (□) periodontal treatment, in unstimulated (a) and stimulated (b) saliva from patients with chronic periodontitis. Bars represent mean ± SEM. **Significantly different from before periodontal treatment (paired Student’s t test). Lower panel: salivary concentration of total nitrates and nitrites at the first evaluation (■) and at the second evaluation (□) performed 3 months later, in unstimulated (c) and stimulated (d) saliva from healthy subjects.

not only from NO formed in periodontal pockets but from nitrates secreted by salivary glands.

Conclusion

Total salivary nitrates and nitrites are increased in saliva from patients with periodontal disease. Because nitrate was described as the basis of a non-immune system-mediated mechanism of defense [29], against gastrointestinal and oral pathogens in animals and humans, its increase in patients with periodontal disease could be considered to be associated with the host defence reaction, although further studies are needed to confirm this hypothesis.

Acknowledgment

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References

