Clinica Chimica Acta 444 (2015) 293–296

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Salivary, blood and plasma nitrite concentrations in periodontal patients and healthy individuals before and after periodontal treatment

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ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 13 February 2015
Accepted 18 February 2015
Available online 5 March 2015

Keywords:
Periodontal disease
Saliva
Blood
Nitric oxide
Nitrite
Chemiluminescence

ABSTRACT

Background: To date, no study has employed ozone-based reductive chemiluminescence to compare nitrite concentration in the saliva of periodontal disease (PD) and healthy individuals or in the various blood compartments of the same individuals before and after periodontal treatment. We evaluated nitrite concentrations in whole, submandibular, and parotid saliva, as well as in whole blood, erythrocytes, and plasma of healthy volunteers and patients with chronic periodontitis.
Methods: Data obtained for the PD and control groups were compared before and 3 months after periodontal therapy.
Results: At baseline, stimulated whole saliva nitrite concentration was lower in PD patients (mean = 57.3 ± 9.8 μmol/L) as compared with healthy individuals (92.5 ± 13.6 μmol/L, $P < 0.05$). PD and periodontal treatment did not affect submandibular or parotid saliva nitrite concentrations. PD patients presented higher baseline whole blood nitrite concentration (238.4 ± 45.7 μmol/L) as compared with values recorded 3 months after therapy (141.3 ± 20.1 nmol/L, $P < 0.05$). PD patients' erythrocytes exhibited higher baseline nitrite concentration (573.1 ± 97.8 nmol/L) as compared with three months after therapy (298.7 ± 52.1 nmol/L, $P < 0.05$). Again, PD and PD treatment did not impact plasma nitrite concentration.
Conclusions: PD patients had lower nitrite concentration in whole saliva, and this situation remained unchanged after periodontal treatment. Nevertheless, erythrocytes and whole blood nitrite levels diminished after periodontal treatment.

1. Introduction

Periodontal disease (PD) is a chronic bacterial infection of the tooth-supporting tissues. This inflammation may lead to destruction of connective tissue, periodontal ligament, and alveolar bone [1]. Disruption of the balance between proteolytic activity of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) has been highly associated with the progression of periodontal diseases, periodontal pocket formation and bone mass loss [2]. Nitric oxide (NO) production by neutrophils and other inflammatory cells has an important anti-microbial activity and, therefore, it can affect many immunological functions [3]. Moreover, NO may increase or decrease MMP activity and expression depending on its concentration, or be affected by the cell- and environment-specific interplay [4]. Thus, the balance between reactive oxygen species and anti-oxidant systems is very important to avoid NO deleterious effects [5], like peroxynitrite production, which may enhance MMP activity, and promote S-nitrosylation of proteins and tissue damage [6].

PD is related with increased inflammatory biomarker levels in saliva [7] and plasma [8]. Moreover, an association between PD and endothelial dysfunction seems to exist as a result of impaired NO bioavailability, increased platelet aggregation, and higher risk of thrombus formation [9].

Literature papers on NO concentration in saliva of PD patients and healthy volunteers have reported substantially different data. Whereas some authors have found higher nitrite + nitrate (NOx) concentration in unstimulated and stimulated saliva of PD subjects [10,11], other authors have detected lower NOx concentration in saliva of PD subjects [12,13]. Moreover, PD patients have been described to display increased plasma NOx concentration [14,15]. All the studies published to date have used the Griess method to infer NO levels. However, no

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http://dx.doi.org/10.1016/j.cca.2015.02.045
0009-8981/

investigation has employed chemiluminescence to analyze NO and compare nitrite concentrations in saliva and blood of PD patients and healthy volunteers in the same study.

2. Materials and methods

The Institutional Review Board on Research involving Human Subjects of the School of Dentistry of Ribeirão Preto approved this investigation. Results obtained with this same cohort on how periodontal therapy affects inflammatory parameters as well as MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2, and myeloperoxidase in plasma, saliva, and crevicular fluid have been published elsewhere [16–20].

Patients were selected and were submitted to a non-surgical periodontal treatment as described by Marcaccini et al. [20]. The subjects included in the PD group (n = 22) were submitted to a non-surgical periodontal treatment, which consisted of four sessions of scaling and root planning over a maximum of four weeks. All the patients, belonging to the PD group or to the healthy group (n = 16), were re-examined every 15 days, for three months. Saliva and blood were collected at baseline and after three months.

Subjects were asked to come for blood collection after a twelve-hour fast. They had their venous blood placed into two vacuum tubes containing heparin. For whole blood samples, a nitrite preserving solution was added to the tube (0.8 M $K_3[Fe(CN)_6]$ and 10% (v/v) Nonidet P-40, at a 1:5 ratio) [21]. For plasma and erythrocyte samples, the tubes were centrifuged at 3000 rpm for 3 min, at room temperature; plasma was collected and stored, and the decanted erythrocytes were mixed with the nitrite preserving solution at a 1:5 ratio.

Stimulated whole saliva was collected by expectoration after the participants had chewed paraffin, to collect clean submandibular saliva. Saliva from the parotid gland was then collected using a modified Carlson–Crittenden collector as described in detail before [22]. All saliva samples were centrifuged at 700 *g* for 15 min, at 4 °C, to pellet the cells without lysis. Next, the supernatant of this first centrifugation was centrifuged a second time at 12,000 *g* for 10 min, at 4 °C, to remove insoluble debris from the suspension. Blood and saliva samples were collected between 7 and 9 a.m.; they were stored at −80 °C until use.

Nitrite concentrations in the samples were determined using a previously described ozone-based reductive chemiluminescence assay [23]. First, saliva samples were diluted in ultrapure water, to obtain a 5% solution of stimulated whole saliva and 50% solution of submandibular and parotid saliva. Then, 100 μL of saliva samples or 300 μL of undiluted whole blood, plasma, or erythrocyte samples was injected into a purge vessel filled with 8 mL of triiodide solution (1.3 g of iodine and 2 g of potassium iodide dissolved in 40 mL of water and 140 mL of acetic acid). The triiodide solution reduces nitrites to NO gas, which is detected by a gas phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer). Data with Gaussian distribution were reported as the mean ± error. Statistical analyses were performed using paired *t* test or unpaired *t* test, as appropriate.

3. Results

Non-surgical periodontal treatment markedly improved all the clinical parameters in both groups (data were published elsewhere) [20]. Stimulated whole saliva had the highest nitrite concentration (Fig. 1A), followed by submandibular saliva (Fig. 1B) and parotid saliva (Fig. 1C). Stimulated whole saliva nitrite concentration was lower in the PD group before and after treatment as compared with the control group at baseline (Fig. 1A, $P < 0.05$ for both). Fig. 1B and C revealed that no statistical differences existed between the groups in terms of submandibular or parotid saliva nitrite concentrations, respectively ($P > 0.05$ in both cases).

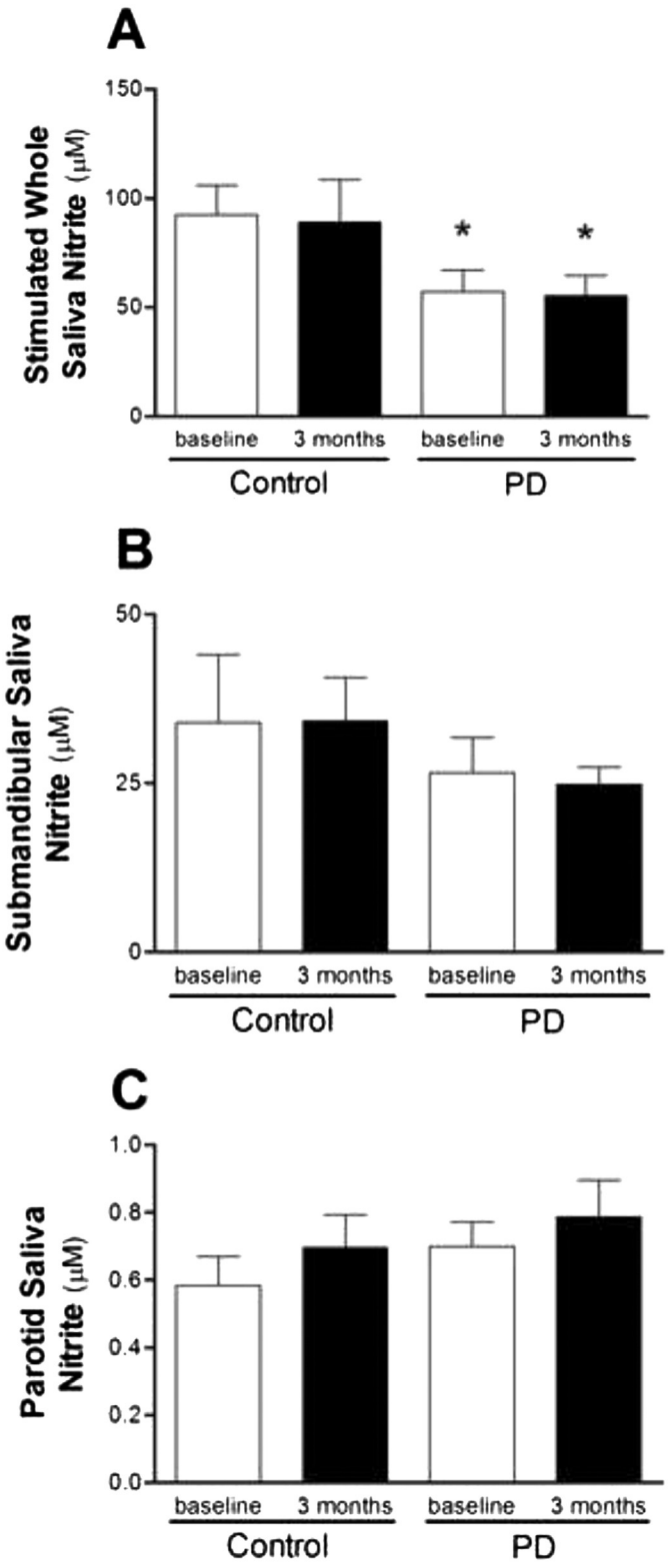

Fig. 1. Nitrite concentration in saliva samples of control and PD groups. (A) Stimulated whole saliva nitrite concentration is lower in the PD group before and after treatment as compared with the control group at baseline ($P < 0.05$ for both cases). PD and periodontal treatment did not affect (B) submandibular or (C) parotid saliva nitrite concentrations ($P > 0.05$ for all comparisons). PD: periodontal disease. Data are expressed as means ± SEM. *$P < 0.05$ versus control at the baseline.

Erythrocytes displayed the highest nitrite concentration (Fig. 2B), followed by whole blood (Fig. 2A) and plasma (Fig. 2C). Treatment reduced whole blood and erythrocyte nitrite concentrations in the PD group