Original Research

Effects of non-surgical periodontal treatment on the L-arginine-nitric oxide pathway and oxidative status in platelets

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Abstract

Several studies have suggested an increase of cardiovascular disease (CVD) risk on periodontitis patients. An enhancement has been demonstrated on both platelet activation and oxidative stress on periodontitis patients, which may contribute for this association. Therefore, the aim of this study was to evaluate the effects of non-surgical periodontal treatment on the L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway and oxidative status in platelets. A total of eight periodontitis patients and eight controls were included in this study. Clinical, laboratory and experimental evaluations were performed on baseline and 90 days after periodontal treatment (except for western blot analysis). The clinical periodontal evaluation included measurements of probing pocket depth (PPD), clinical attachment loss (CAL), % of sites with plaque and % of sites with bleeding on probing. We evaluated: L-[^{3}H]arginine influx; nitric oxide synthase (NOS) and arginase enzymes activity and expression; expression of guanylate cyclase and phosphodiesterase-5 enzymes; cGMP levels; platelet aggregation; oxidative status through superoxide dismutase (SOD) and catalase activities, and measurement of reactive oxygen species (ROS) levels and C-reactive protein (CRP) levels. The initial results showed an activation of both L-arginine influx and via system y^+L associated with reduced intraplatelet cGMP levels in periodontitis patients and increased systemic levels of CRP. After periodontal treatment, there was a significant reduction of the % of sites with PPD 4–5mm, % of sites with CAL 4–5 mm, and an enhancement in cGMP levels and SOD activity. Moreover, CRP levels were reduced after treatment. Therefore, alterations in the intraplatelet L-arginine-NO-cGMP pathway and oxidant–antioxidant balance associated with a systemic inflammatory response may lead to platelet dysfunction, which may contribute to a higher risk of CVD in periodontitis.

Keywords: L-arginine-NO-cGMP pathway, platelets, periodontitis, oxidative status

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Introduction

Cardiovascular diseases (CVDs) are the leading causes of death in the world, being responsible for more than 17 million deaths in 2008. A large proportion of CVDs result from atherosclerosis, a complex pathological process in the walls of blood vessels characterized for deposition of fatty materials and cholesterol inside the lumen of medium- and large-sized blood vessels. The rupture of this plaque triggers the formation of a blood clot, which can cause a heart attack or a stroke if it blocks the coronary artery or a blood vessel in the brain, respectively.[1] Since the traditional risk factors for atherosclerosis were not sufficient to account for the aetiology of this multifactorial process, several novel risk factors have been identified, such as microbial pathogens, fibrinogen, and C-reactive protein (CRP).[2]

In this context, over the last two decades, the relationship between periodontitis and CVDs has been investigated. However, the physiopathological mechanisms involved in this association are still not fully understood.[3–7] Periodontitis is stated as a chronic inflammatory process, mainly associated with Gram negative bacteria, characterized by the loss of connective tissue attachment and bone around the teeth, whose prevalence in the adult population is about 40–50%.[8] Since only a small proportion of the population develops periodontitis in severe forms, it is possible that the initiation and progression of the disease are the result of activation of the host's immune-inflammatory

response to the bacterial challenge.[9–11] Periodontitis may increase levels of inflammation markers such as CRP, fibrinogen, interleukin (IL) 6, IL 1, and tumour necrosis factor-alpha (TNF-α), interferon gamma (IFNγ) which correlates to cardiovascular events.[3,12,13] Studies *in vitro* and *in vivo* showed that these cytokines are capable of altering platelet function.[14–16] Moreover, the presence of endothelial dysfunction was demonstrated, associated with an impairment of nitric oxide (NO) activity and platelet activation in periodontitis patients.[17–19]

NO exerts important protective actions on cardiovascular system, through the regulation of vascular tone and endothelium-dependent vasodilatation. With regard to effects on platelet function, the NO produced by both endothelial cells and platelet inhibits the processes of platelet adhesion, activation, and aggregation via the soluble guanylate cyclase (sGC) enzyme, a heterodimeric enzyme which catalyses the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), a second messenger, which diminishes platelet cytosolic calcium concentration (Ca^{2+}). Therefore, NO reduces monocyte–platelet aggregates in the circulation, which are believed to play an important role in the initiation and progression of atherosclerosis.[20] The cGMP is a temporary signalling molecule, since it is inactivated by a group of enzymes called phosphodiesterases. On the relation between NO and cGMP, probably, the most important is phosphodiesterase 5.[21]

The NO is synthesized from nitric oxide synthase (NOS), which converts the essential conditional amino acid L-arginine into L-citrulline and NO. Platelets possess the endothelial (eNOS) and inducible (iNOS) isoforms of NOS. Although the intracellular L-arginine levels are enough to reach the enzyme Michaelis constant, L-arginine influx is essential to NO production, which is known as the 'L-arginine–NO pathway paradox'. Previously, our group has demonstrated that L-arginine influx in platelets is mediated by the transport system y^+L.[22] NOS enzymes can also produce superoxide anion (O_2^-), a reactive oxygen species (ROS). It can be observed under reduced levels of L-arginine (substrate) or tetrahydrobiopterin (BH_4, an cofactor).[23]

L-arginine participates in different metabolic pathways, such as the urea cycle. This amino acid is a substrate for the enzyme arginase, which hydrolyses L-arginine into L-ornithine and urea. Therefore, the NO levels can be regulated by the competition between NOS and arginase enzymes.[24]

In addition, NO bioavailability is also regulated by this reaction with O_2^- to produce the nitrogen reactive species peroxynitrite ($ONOO^-$), which can lead to lipid peroxidation and protein and DNA damage through oxidation and/or nitration of cellular components.[25] Several studies suggest that periodontitis is associated with increased lipid peroxidation[26,27] and oxidative DNA and protein damage[27–31]; and alterations in antioxidant defences.[26,32–36] Moreover, there is evidence that periodontal treatment is able to reduce oxidative injury and improve antioxidant defence.[27,32,37]

The aim of the present study was to investigate, in detail, the L-arginine-NO-cGMP pathway and oxidative stress parameters in platelets of periodontitis patients, and the effect of non-surgical periodontal treatment in these variables.

Materials and methods

Study population

A total of eight patients with periodontitis (44.5 ± 1.2 years) and eight controls (49.5 ± 2.1) were included in the study. These patients were recruited among subjects seeking dental treatment at the Rio de Janeiro State University or at the Dentistry Institute at the Catholic University of Rio de Janeiro. The inclusion criteria for the periodontitis group was the presence of at least three teeth with a clinical attachment loss (CAL) ≥ 4 mm and a probing pocket depth (PPD) ≥ 5 mm.[38] To be included in the control group, the patients should have no PPD > 3 mm and no interdental attachment loss.[38] A minimum of 12 teeth, excluding the third molars, was an inclusion criterion for both groups. Exclusion criteria were the presence of systemic disease, pregnancy, periodontal treatment in the previous six months, and recent use of antibiotics, anti-inflammatory drugs (in the preceding three months for both), and antiplatelet drugs (within the last 10 days). This study respected the principles outlined in the Declaration of Helsinki and was approved by the Pedro Ernesto University Hospital Ethical Committee (2269-CEP/HUPE). Informed written consent was obtained from all subjects.

Periodontal examination

The periodontal examination included: percentage of sites with visible supragingival plaque, recorded as present or absent; percentage of sites with bleeding on probing (BOP), recorded as present or absent; PPD, and CAL. PPD and CAL were performed in six sites per tooth (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, and disto-lingual), whereas the % of visible supragingival plaque and BOP was evaluated in four sites/tooth (mesial, buccal, distal, and lingual). The examinations were performed by the same calibrated examiner (kappa = 0.76), using a calibrated periodontal probe (UNC-15, Hu-Friedy, Chicago, IL, EUA).

Non-surgical periodontal treatment

Periodontal therapy included oral hygiene instructions, supra and subgingival scaling, and extractions of teeth with poor prognosis. The periodontal treatment was performed by using an ultrasonic device (Cavitron Select, Dentsply, NY) and Gracey curettes (Hu-Friedy, Chicago, IL). The periodontitis patients were recalled 90 days after treatment[39] to repeat clinical, laboratorial, and experimental analysis.

Blood collection and preparative procedures

Venous blood was anticoagulated with a citric acid-dextrose anticoagulant (mmol) (73.7 mM citric acid, 85.9 mM trisodium citrate, and 111 mM dextrose). As described previously, platelet-rich plasma (PRP), obtained

by centrifugation (Eppendorf Centrifuge 5804 R, Germany) of whole blood at 200 *g* for 15 min, was centrifuged at 900 *g* for 10 min. Pellet was resuspended in Kreb's buffer (mmol) (119 NaCl, 4.6 KCl, 1.5 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 15 $NaHCO_3$, and 11 glucose, pH 7.4).[40] Platelets were counted by using the cell counter Automated Hematology Analyzer XT-1800i (Sysmex Corporation, Kobe, Japan) and the leucocyte contamination was minimal (less than 0.01×10^6/mL).

L-arginine influx protocol

For influx experiments, 0.5 mL aliquot of resuspended platelets (1×10^9 platelets/mL) was incubated with L-[^{3}H]-arginine (1–50 μmol/L) at 37°C for 5 min. Total transport was fractionated into diffusion and system y^+L by *cis*-inhibition with unlabelled amino acid L-leucine (10 mmol/L). Transport was terminated by rapid centrifugation (Centrifuge 5417 C, Eppendorf AG, Germany), followed by two washes with Kreb's buffer, re-centrifugation and lysis with Triton for β-scintillation counting (LS 6500 Liquid Scintillation Counter, Beckman Counter Inc., CA) arginase.[24]

Measurement of NOS activity

Basal NOS activity was determined from the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline. Platelet suspensions were incubated at 37°C in the presence of L-[^{3}H]-arginine (37 kBq/mL) plus unlabelled L-arginine (1 mmol/L) for 45 min. All reactions were stopped by rapid centrifugation (2000*g*,15 s) (Centrifuge 5417 C, Eppendorf AG, Germany), followed by two washes with Kreb's buffer. The platelet pellet was lysed with 0.1% Triton. Samples were applied to a Dowex cation exchange resin column. The L-[^{3}H]-citrulline was eluted with 2 mL water and radioactivity was measured by liquid scintillation counting (LS 6500 Liquid Scintillation Counter, Beckman Counter Inc., CA).[26]

Evaluation of arginase activity

Basal arginase activity was measured in platelet lysates by the conversion of [^{14}C]-L-arginine into [^{14}C]-urea. Briefly, platelets were isolated by centrifugation (2000*g*, 4°C, 15 min) (Eppendorf Centrifuge 5804 R, Germany) and the pellet was resuspended in lysis buffer composed of 50 mmol/L Tris-HCl, 10 mmol/L CHAPS, 2 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), 1 mmol/L pepstatin A and 2 mmol/L leupeptin (pH 7.4). Cells were sonicated (MaxiClean 1400, Unique, São Paulo, Brazil) and the homogenate was centrifuged (Centrifuge 5417 C, Eppendorf AG, Germany) at 14,000 *g* for 10 min at 4°C. Aliquots of platelet lysates were incubated for 2 h at 37°C in a buffer containing 9 mmol/L Tris-HCl and 1 mmol/L MnCl2 (pH 9.6) in the presence of 100 μmol [^{14}C]-L-arginine (3 kBq/mL). The reaction was stopped by the addition of ice-cold stop buffer (250 mmol/L sodium acetate and 100 mmol/L urea). Samples were applied to a Dowex cation exchange resin column and radioactivity was measured by a liquid scintillation counter (LS 6500 Liquid Scintillation Counter, Beckman Counter Inc.).[24]

Western blot analysis for eNOS, iNOS, arginase II, sGC and PDE 5

Platelets isolated from the PRP by centrifugation (Eppendorf Centrifuge 5804 R, Germany) were washed and lysed with lysis buffer. Protein will be quantified using BCA protein assay reagent. Samples containing 20 μg protein will be prepared in equal volumes of sample application buffer (NuPage LDS Sample Buffer, Invitrogen, Carlsbad, CA), separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes and then immunoblotted with mouse monoclonal antibodies against human eNOS, iNOS, arginase II, PDE 5, and α_1 and β_1 subunits of sGC (Santa Cruz Biotechnology, CA). Anti-rabbit β-tubulin (Santa Cruz Biotechnology, CA) was used as quality control of protein quantity. The bands detection was performed by incubation with ECL (Amersham-Biosciences, Buckinghamshire, UK). Full-Range Rainbow™ Molecular Weight Marker (GE Healthcare, USA) was used as standard molecular weight and lysates of endothelial cells (eNOS), macrophages (iNOS), and kidney (other analysis) (Santa Cruz Biotechnology, CA) were employed as positive controls. The bands intensity was normalized by intensity of correspondent β-tubulin band. The images quantification was performed by using ImageJ program (ImageJ1.43 μ, National Institutes of Health, USA).

Protein quantification

Protein was measured through colorimetric assay, using the BCA™ Protein Assay (Thermo Fisher Scientific Pierce Protein Research, IL).[24]

Determination of platelet cGMP levels

cGMP content was determined in washed platelets using a commercial enzyme-linked immunosorbent assay (ELISA) method (Cyclic GMP EIA kit, Cayman Chemical Company, MI). Briefly, the aliquots of the platelet suspension were preincubated with 200 μM isobutylmethylxanthine (IBMX, a phosphodiesterase inhibitor) for 30 min. Ice-cold perchloric acid (0.3 mol/L) was added to the platelet suspension and the platelets were lysed by sonication (MaxiClean 1400, Unique, São Paulo, Brazil), followed by rapid freezing in liquid nitrogen. Cell debris was then pelleted by centrifugation (2000*g*, 20 min) (Centrifuge 5417 C, Eppendorf AG, Germany). The supernatants containing cGMP were collected and stored at −80°C (Thermo Scientific Forma 900 series, USA) for subsequent analysis by ELISA (TP READER NM, Thermo Plate, Rayto Life and Analytical Sciences C. Ltd., Germany).[24]

Platelet aggregation assay

Platelet aggregation was evaluated on PRP by optical densitometry. Briefly, blood samples were anticoagulated with 3.8% trisodium citrate and centrifuged (Eppendorf

Centrifuge 5804 R, Germany) at 200 *g* for 15 min at room temperature. Platelet-poor plasma (PPP) was obtained by centrifuging (Eppendorf Centrifuge 5804 R, Germany) the leftover blood at 900 *g* for 10 min. The platelet concentration in PRP was adjusted with PPP to a constant count of 1.5×10^8/mL. Aggregation was induced by collagen (4 μg/mL) and responses monitored for 5 min in a four-channel aggregometer (Chrono-Log, Havertown, PA). Tests were performed at 37°C with a stirring speed of 900 rpm. Maximal aggregation was expressed in percentage.[24]

Measurement of platelet ROS by nitrotetrazolium blue

This method is based on the reduction of the nitrotetrazolium blue to formazan by ROS. Approximately 90% of this salt react with superoxide anion, but it can also react with other reactive species including hydroxyl radical, hydrogen peroxide (H_2O_2) and hypochlorous acid.[41] The solution of 0.1% nitrotetrazolium blue was added to 100 μL of phosphate buffer (PBS) and 100 μL of platelet suspension, followed by incubation at 37°C for 1 h. The reaction was interrupted by centrifugation (Centrifugue 5417 C, Eppendorf AG, Germany) at 14,000 rpm for three minutes. The supernatants were discarded and 500 μL of PBS was added. This procedure was repeated and, finally, 200 μL of KOH (2 M) and dimethylsulfoxide (DMSO) solution were added, the samples solubilized, and evaluated through ELISA method (TP READER NM, Thermo Plate, Rayto Life and Analytical Sciences C. Ltd., Germany). The analyses were performed in duplicate.

Evaluation of SOD activity

In three different cuvettes, was added 20, 40, and 60 μL of platelet suspension plus 20 μL of catalase (2.4 mg/mL distilled water), 1940 μL of glycine buffer (0.75 g/ 200 mL distilled water; pH 10.2) and 40 μL of norepinephrine (19 mg/mL distilled water + 15 μL de HCl concentred). The enzyme activity was measured via kinetic analysis for 180 s, with readings at intervals of 10 s, at a wavelength of 480 nm (Ultrospec™ 2100 pro UV/Visible Spectrophotometer, GE Healthcare, Uppsala, Sweden).[42]

Measurement of catalase activity

Catalase is a hemeprotein which catalyses the H_2O_2 degradation. In a quartz cuvette, was added 200 μL of platelet suspension plus 1800 μL of H_2O_2 buffer (25 mL of PBS: 40 μL of H_2O_2). The reading was performed at 0, 30 and 60 s, at a wavelength of 240 nm (Ultrospec™ 2100 pro UV/Visible Spectrophotometer, GE Healthcare, Uppsala, Sweden).[42]

Determination of CRP protein levels

A highly sensitive turbidimetric test was performed to determine the serum levels of CRP.[43]

Statistical analysis

The normally distributed data were presented as mean and standard error (SE), while the non-normally distributed data were presented as median, with minimum and maximum. The unit of evaluation was the individual and the statistic significance was determined when $P \leq 0.05$. The dependent and independent Student t-tests were used for normally distributed data, whereas Wilcoxon and Mann–Whitney tests were used for dependent and independent analysis, respectively, of non-parametric data. The program Prisma 5.0 was used to perform statistic analysis (GraphPad Prism Program, San Diego, CA).

Results

There was no significant difference among the groups in terms of age, gender, body mass index (BMI), and systolic and diastolic blood pressures, and laboratory parameters (Table 1).

As demonstrated in Table 2, after periodontal treatment there was a significant reduction in the % of sites with supragingival plaque, as compared to the control group. Moreover, the % of sites with PBS 4–5 mm and CAL 4–5 mm also decreased significantly.

L-arginine influx in platelets

At the begining of the study, total L-arginine transport and via the y^+L system were increased in periodontitis patients compared to the controls. After periodontal treatment, a reduction was seen in L-arginine influx through the y^+L system to levels comparable to those observed in the control group (Figure 1).

Basal NOS activity

There was no significant difference in basal NOS activity (pmol/10^8 cells/min, $n = 8$) among controls (0.12 ± 0.02) and periodontitis patients before (D0: 0.11 ± 0.02) and after periodontal treatment (D90: 0.07 ± 0.02).

Arginase activity

No significant differences were found among the groups at the beginning of the study (controls: 40.1 [6.1–464.0] pmol urea/mg protein/2 h, $n = 8$ vs. periodontitis D0: 21.7 [4.2–49.9] pmol urea/mg protein/2 h, $n = 7$) and after periodontal treatment (periodontitis D90: 84.2 [16.5–170.6] pmol urea/mg protein/2 h, $n = 7$).

Western blot analysis for eNOS, iNOS, arginase II, sGC and PDE 5

There were no significant differences between the groups on the platelet expression of eNOS (controls: 0.9 ± 0.4 vs. periodontitis D0: 1.9 ± 0.6 arbitrary units, $n = 3$), iNOS (controls: 0.6 ± 0.2 vs. periodontitis D0: $1.6 \pm 0{,}6$ arbitrary units, $n = 3$), arginase II (controls: 30.3 ± 7.3 vs. periodontitis D0: 54.3 ± 15.7 arbitrary units, $n = 3$), α_1 (controls: 21.3 ± 4.3 vs. periodontitis D0: 53.9 ± 18.9 arbitrary units, $n = 3$) and β_1 (controls: 25.5 ± 5.5 vs. periodontitis D0: 68.0 ± 35.8 arbitrary units, $n = 3$) GCs subunits, and PDE 5 (control/gingivitis: 15.6 ± 9.1 vs. periodontitis D0: 29.0 ± 12.6 arbitrary units, $n = 3$). Figure 2 illustrates the enzymatic expression

Table 1 Demographic characteristics (age and gender), BMI, arterial blood pressure, and laboratorial data from control ($n = 8$), periodontitis D0 ($n = 8$), and periodontitis D90 ($n = 8$) groups, demonstrated as media ± SE or median (min-max)

Data	Control	Periodontitis D0	Periodontitis D90
Age (years)	44.5 ± 1.2	49.5 ± 2.1	49.5 ± 2.1
Gender (*n*; W/M)	4/4	5/3	5/3
BMI (kg/m^2)	24.5 ± 1.6	26.5 ± 1.9	26.1 ± 1.7
SBP (mmHg)	117.6 ± 4.7	118.4 ± 5.1	120.9 ± 3.5
DBP (mmHg)	71.6 ± 3.8	74.8 ± 3.3	74.8 ± 3.3
Leukocytes (10^3/mm^3)	7.0 ± 0.7	5.7 ± 0.6	5.6 ± 0.6
Glucose (mg/dL)	82.0 (75–95)	88.5 (44–106)	83.5 (68–95)
Total cholesterol (mg/dL)	189.9 ± 14.3	209.3 ± 16.4	208.5 ± 12.4
HDL cholesterol (mg/dL)	44 (42–56)	51.5 (32–67)	47 (39–75)
LDL cholesterol (mg/dL)	114.5 ± 17.8	138.8 ± 15.5	143.4 ± 11.9
Trygliceride (mg/dL)	79 (27–212)	99 (53–143)	71.5 (55–167)
CRP (mg/dL)	0.2 ± 0.07	1.2 ± 0.39*	0.9 ± 0.33

SBP: systolic blood pressure; DBP: dyastolic blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; CRP: C reactive protein.
*$P < 0.05$ – vs. control.

Table 2 Number of teeth and clinical periodontal variables (% of sites; mean ± SE or median [min-max]) in control, periodontitis D0, and periodontitis D90 groups ($n = 8$)

	Control	Periodontitis D0	Periodontitis D90
Teeth (*n*)	23 (17–28)	24 (19–27)	24 (19–25)
Visible plaque	30.3 ± 8.0	17.1 ± 3.7	12.8 ± 1.6*
BOP	21.6 ± 7.9	29.4 ± 4.3	22.9 ± 5.4
PPD 4–5 mm	–	11.8 (3.5–25.0)	5.8 (2.1–16.7)†
PPD ≥ 6 mm	–	7.6 ± 2.8	2.0 ± 1.2
CAL 4–5 mm	–	16.8 ± 2.2	13.1 ± 1.5†
CAL ≥ 6 mm	–	10.8 ± 4.5	5.3 ± 2.4

BOP: bleeding on probing; PPD: probing pocket depth; CAL: clinical attachment loss.
*$P < 0.05$ – vs. control.
†$P < 0.05$ – vs. periodontitis D0.

by western blotting analysis in controls and in periodontitis D0 groups.

Platelet cGMP content

At the begining of the study, basal cGMP levels in platelets from periodontitis patients were significantly decreased compared to controls. After periodontal therapy, the cGMP levels were signicantly higher than those observed in these patients at the beginning of the study and without significant differences when compared to controls (Figure 3).

Platelet aggregation

In platelet aggregation assays (%) induced by fibrillar collagen (4 μg/mL), there was no significant difference between the groups (control: 78.9 ± 4.5, $n = 8$; periodontitis D0: 83.3 ± 5.1, $n = 8$; periodontitis D90: 86.1 ± 3.4, $n = 8$) (Figure 4).

Measurement of platelet ROS by nitrotetrazolium blue

We did not find any significant difference among the groups in ROS platelet levels (controls: 0.001 ± 0.0002 μg of formazan/μg protein, $n = 8$; periodontitis D0: 0.0008 ± 4.76 e-005 μg of formazan/μg protein, $n = 8$; periodontitis D90: 0.0008 ± 6.21 e-005 μg of formazan/μg protein, $n = 8$).

SOD activity

At the begining of the study, SOD activity did not differ between periodontitis patients and controls, but it increased after periodontal treatment, when compared to the values observed for periodontitis patients on D0. There was no significant difference between controls and periodontitis D90 group (Figure 5).

Catalase activity

There were no significant differences in platelet catalase activity among the groups (controls: 0.16 [0.02–0.22] U of catalase/mg protein, $n = 8$; periodontitis D0: 0.16 [0.10–0.68] U of catalase/mg protein, $n = 8$; periodontitis D90: 0.23 [0.11–0.79] U of catalase/mg protein, $n = 8$).

Levels of CRP

At the begining of the study, the levels of CRP were significantly increased in periodontitis patients compared to controls. After periodontal treatment, there was not a significant reduction in these levels (Table 1).

Discussion

This study investigated, for the first time, the L-arginine-NO-cGMP pathway and the oxidative status in platelets from periodontitis patients and the effects of non-surgical periodontal therapy. Our results showed an increase in total L-arginine uptake and via transport system y^+L, substrate supply for NOS, in patients with periodontitis at baseline,

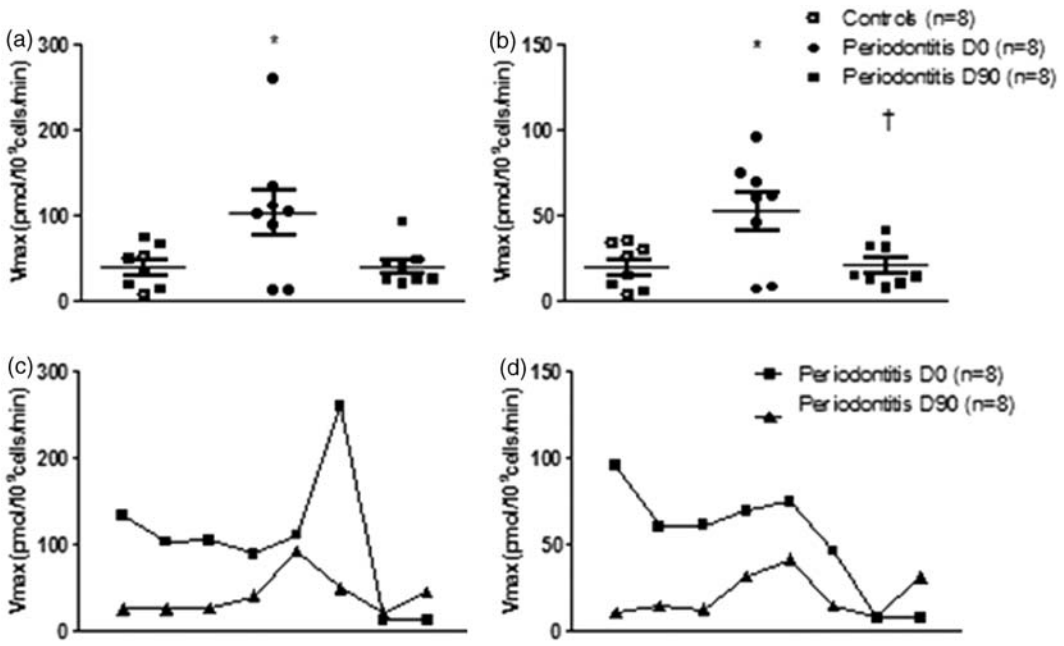

Figure 1 (a) Total L-arginine influx and (b) via y^+L system in platelets from control, periodontitis D0, and periodontitis D90 groups. (c) Line diagrams of total L-arginine influx and (d) via y^+L system in platelets from periodontitis patients pre- and post-treatment. In Figure 1A and B, values denote means ± SE. $^*P < 0.05$ vs. control (independent Student t-test) and $^\dagger P < 0.05$ vs. periodontitis D0 (Wilcoxon signed-rank test)

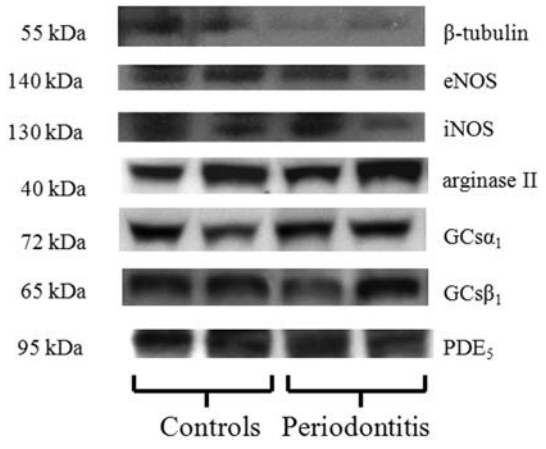

Figure 2 Illustration of enzymatic expression by western blotting analysis in control and in periodontitis D0 groups

which was reversed by periodontal treatment. Moreover, the periodontal therapy leads to an enhancement on cGMP levels to comparable values to those observed on individuals without periodontitis, accompanied by higher SOD activity.

In spite of the role of NO on periodontitis has been investigated, there is no study about L-arginine transport, an essential step for NO synthesis. Besides, there is few information regarding the modulation of system y^+L, despite its wide detection in different cell types such as erythrocytes, kidneys, bowel, placenta and platelets.[40] It is well established that cell membrane fluidity may interfere in transport activity, since the proteins are immersed in the lipid bi-layer.[44] The activation of system y^+L observed in this study may be the result of a modification of platelet membrane fluidity by oxidative stress.[45] This hypothesis may be corroborated by the normalization of y^+L system activity after periodontal treatment, accompanied by an enhancement in the activity of the antioxidant enzyme SOD. Another possibility is the activation of the y^+L system by the inflammatory cytokine IFNγ, as demonstrated by Rotoli *et al*.[46] in human monocytes and that the levels of this cytokine may be increased in periodontitis.[47]

The NOS and arginase activities were poorly studied on periodontitis patients (gingival biopsies, saliva samples, and peripheral neutrophils) and there is not a consensus about the activity of these enzymes.[48–50] Although the L-arginine influx in platelets from periodontitis patients was increased in our study, the NOS activity and expression were similar to those observed in platelets from patients without periodontitis, as also the activity and expression of arginase. These results point to an equal production of NO by NOS in all groups. However, the levels of the second messenger of NO physiological function cGMP were decreased in platelets from periodontitis patients on the baseline, similar to Mashayekhi *et al*.[51] observed on saliva samples. The reduction on cGMP levels may be explained by: decreased activity and/or expression of sGC, an enzyme that converts GTP into cGMP, after activation by NO; increased activity and/or expression of PDE, the enzyme responsible for cGMP degradation; and reduced NO bioavailability, due to its reaction with O_2^-. Here, we did not investigate the activity of enzymes sGC and PDE, but we evaluated the expression of them and the

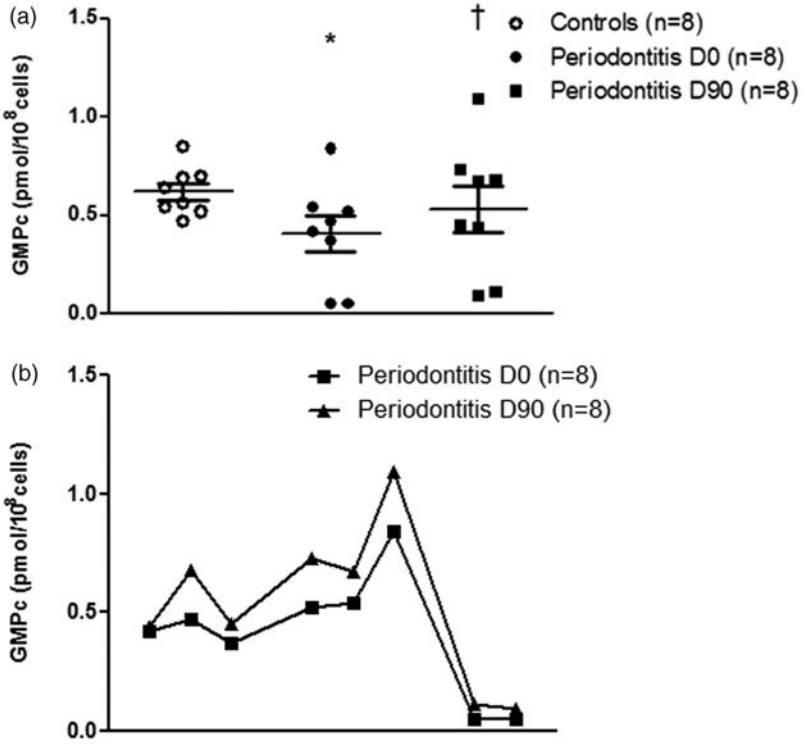

Figure 3 (a) Intraplatelet cGMP levels on control, periodontitis D0 and periodontitis D90 groups. (b) Line diagram of cGMP levels in platelets from periodontitis patients pre- and post-treatment. In Figure 3a, values denote means ± SE of eight patients from each group. $^{*}P < 0.05$ vs. control (independent Student t-test) and $^{\dagger}P < 0.05$ vs. periodontitis D0 (dependent Student t-test)

oxidative stress. Our study did not show any significant difference between periodontitis and controls on day 0 on expression of the enzymes and oxidant status, although there is growing evidence of increased oxidative damage in periodontitis[26–39]. It is important to highlight that we performed only one technique to investigate the oxidative damage and there is divergence if the antioxidant defence is increased or decreased in periodontitis patients.[26,32–36] Moreover, the oxidant status was investigated in platelets from periodontitis patients for the first time in this study.

Among the NO actions in platelets mediated by cGMP, the plasma levels of soluble P-selectin, surface expression of both P-selectin and glycoprotein IIb/IIIa (GPIIbIIIa), and the vasodilator stimulated phosphoprotein phosphorylation (VASP)[18,19,52] have been previously investigated in periodontitis. These studies suggest that platelet activation in this disease is the result of reduced levels of cGMP, which was demonstrated in our study. On the other hand, our experiments did not show increased platelet aggregation in periodontitis. This may be due to the fact that we evaluated only one concentration of an agonist (4 μg/mL of collagen). Moreover, normal levels of platelet aggregation can be maintained by the adenosine monophosphate pathway. In this pathway, human platelets are inhibited by prostacyclin-I2, which directly activates G_s-protein-coupled prostanoid membrane receptors, and thereby increases the intracellular second messenger cyclic adenosine monophosphate (cAMP). Its effects are mediated by cAMP-dependent protein kinases (PKG and PKA), which phosphorylate substrate proteins involved in platelet inhibitory pathways.[53]

The periodontal therapy leads to an improvement on clinical periodontal parameters (the percentage of sites with PPD and CAL 4–5 mm decrease), but there is not a significant reduction on CRP levels. According to the meta-analysis performed by Paraskevas *et al.*,[54] which included only studies with subjects without any systemic disorders, there is strong evidence from cross-sectional studies that plasma CRP levels in periodontitis is elevated compared with controls. However, there is modest evidence on the effect of periodontal therapy lowering the levels of CRP.[54] To our knowledge, after this meta-analysis, only one study investigated the effect of periodontal treatment in systemically health patients. In contrast to our study, Radafshar *et al.*[55] reported that intensive non-surgical periodontal therapy is effective in reducing CRP levels in patients with advanced periodontitis. Differences in terms of disease extension and severity, periodontal treatment protocol, and revaluation period may lead to this divergence.

After periodontal treatment, our study showed an enhancement in intraplatelet cGMP levels, which were similar to those observed in patients without periodontitis and were accompanied by higher SOD activity. SOD is the first-line-of-defence enzyme that eliminates O_2^- by catalysing its dismutation into O_2 and H_2O_2[21] Thus, the increased SOD activity found in this study may result in an improvement in NO bioavailability due to a lower reaction with O_2^- anion, and consequently lead to higher levels of cGMP. In Figure 5B, we can note that SOD data are variable. This variability was also observed by our group in other diseases.[42,43] Another factor which may lead to this result is

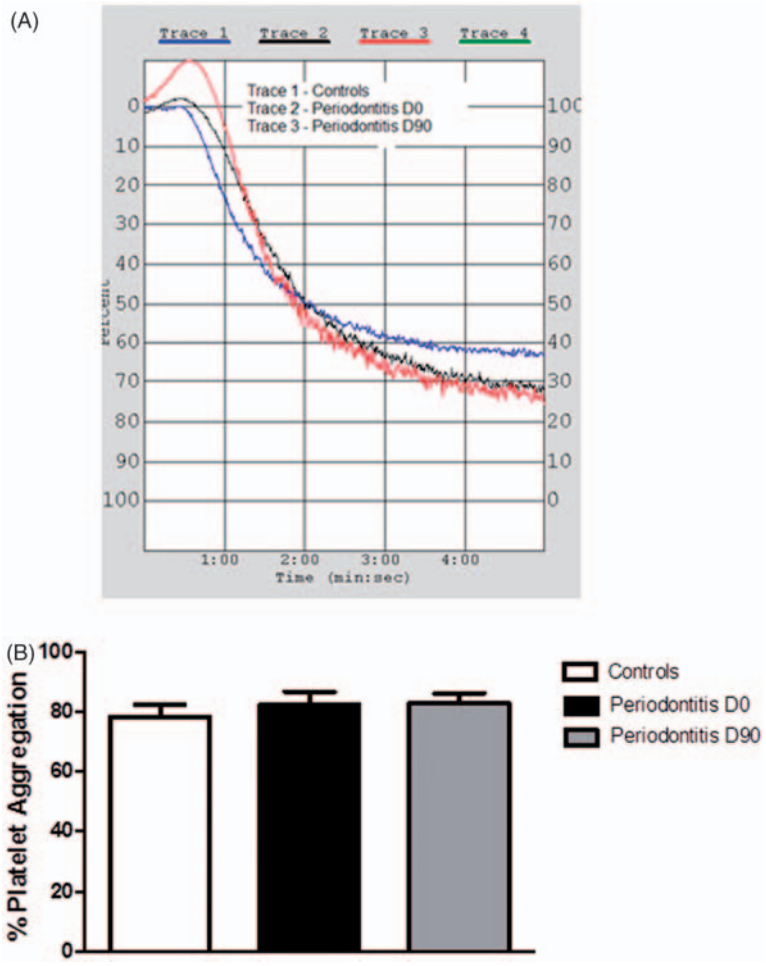

Figure 4 (a) Representative aggregation curves, in presence of collagen 4 μg/mL, of one control, periodontitis D0, and periodontitis patient. (b) Platelet aggregation induced by collagen in the fibrillar form (4 mg/mL) in control, periodontitis D0 and periodontitis D90 groups. Values denote means±SE of eight patients from each group (Student t-test)

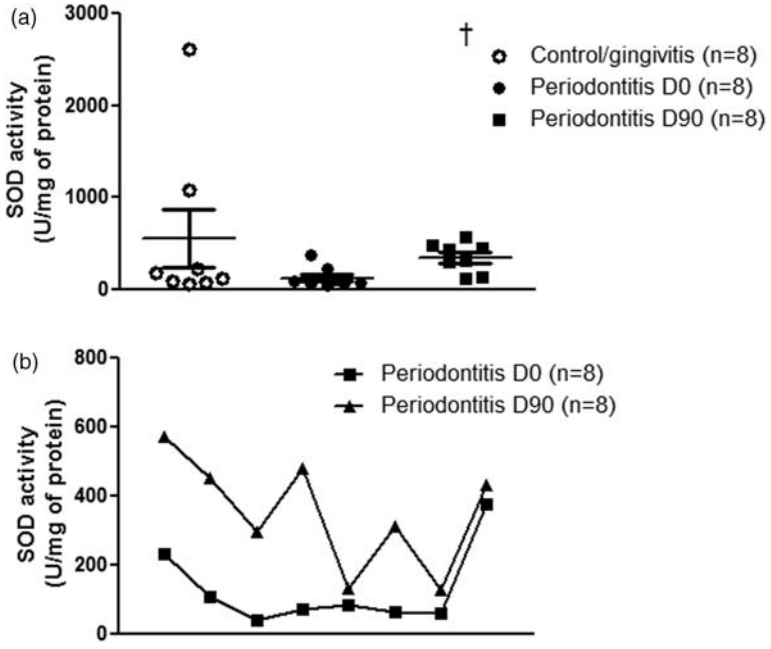

Figure 5 (a) SOD activity in platelets from control, periodontitis D0, and periodontitis D90 groups. (b) Line diagram of SOD activity in platelets from periodontitis patients pre- and post-treatment. In Figure 5A, values denote means ± SE of eight patients from each group. $^{\dagger}P < 0.05$ vs. periodontitis D0 (Wilcoxon test)

the different period of sample storage resultant of the variable periodontal treatment length, which was minimized as much as possible.

In conclusion, this study suggests reduced NO bioavailability in platelets from periodontitis patients and that non-surgical periodontal therapy was effective to revert this condition, through an enhancement in antioxidant defence and cGMP levels. Therefore, alterations in the L-arginine-NO-cGMP pathway and oxidative stress may lead to platelet dysfunction, which could contribute to a higher risk of CVD in periodontitis patients.

Author contribution statement: MAdeSS – designed the study protocol, carried out the clinical assessment, conducted the experiments, analysis and interpretation of these data, and drafted the manuscript.
RGF – designed the study protocol and drafted the manuscript.
NRP – conducted the experiments and analysis and interpretation of these data.
MAM – conducted the experiments and analysis and interpretation of these data.
MBM – conducted the experiments and analysis and interpretation of these data.
ACM-R – designed the study protocol and drafted the manuscript.
CMdSF – designed the study protocol and drafted the manuscript.
TMCB – designed the study protocol and drafted the manuscript.

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