Archives of Oral Biology 63 (2016) 66–74

Contents lists available at ScienceDirect

Archives of Oral Biology

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

Endothelial dysfunction in rats with ligature-induced periodontitis: Participation of nitric oxide and cycloxygenase-2-derived products

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ARTICLE INFO

Article history:
Received 23 June 2015
Received in revised form 6 November 2015
Accepted 29 November 2015

Keywords:
Periodontitis
Endothelium
Vascular
Aorta
Nitric oxide
Cyclooxygenase 2
Inflammation

ABSTRACT

Objectives: Considering the evident relationship between periodontitis and cardiovascular diseases in humans, we aimed to study the in vitro vascular reactivity of aorta rings prepared from rats with ligature-induced periodontitis.
Methods: Seven days after the induction of unilateral periodontitis, the animals were euthanised; rings were prepared from the descending abdominal aortas and mounted in tissue baths for the in vitro measurement of the isometric force responses to norepinephrine (NE) and acetylcholine (ACh), as well as in the presence of inhibitors of nitric oxide synthase (NOS) and cycloxygenase (COX) isoenzymes. Aortic COX and NOS gene expressions were analysed by RT-PCR, as well as protein COX-2 expression by Western blot.
Results: Periodontitis resulted in significant alveolar bone loss and did not affect arterial pressure. However, both NE-induced contraction and ACh-induced relaxation were significantly decreased and related to the presence of endothelium. Diminished eNOS and augmented COX-2 and iNOS expressions were found in the aortas from rats with periodontitis, and the pharmacological inhibition of COX-2 or iNOS improved the observed vasomotor deficiencies.
Conclusions: We can thus conclude that periodontitis induces significant endothelial dysfunction in rat aorta which is characterized by decreased eNOS expression and mediated by upregulated iNOS and COX-2 products.

1. Introduction

Periodontal diseases (PDs) comprise a diverse group of clinical situations in which induction of an inflammatory process results in destruction of the tooth attachment apparatus, reabsorption of supporting alveolar bone and, if untreated, tooth loss (Offenbacher, 1996).

PD is one of the most common diseases of the oral cavity is characterized mainly by Gram-negative bacterial infection, although the presence of some Gram-positive anaerobic bacilli has also been associated with clinical indicators of human PD (Booth, Downes, Van den Berg, & Wade, 2004). The relationship between PD and systemic diseases, such as diabetes (Taylor et al., 1996; Duarte et al., 2014), complications in pregnancy (Michalowicz et al., 2006), rheumatoid arthritis (Mercado, Marshall, Klestov, & Bartold, 2001; De Smit et al., 2015) and cardiovascular disease (CVD) (Genco & Van Dyke, 2010; Beck et al., 2001) has been the focus of numerous reports and reviews (Taylor et al., 1996; Duarte et al., 2014; Michalowicz et al., 2006; De Smit et al., 2015; Genco & Van Dyke, 2010; Beck et al., 2001; Cullinan & Seymour, 2013; Hajishengallis, 2015).

Given the high prevalence of PD, any risk attributable to future CVD is important for public health (El Kholy, Genco, & Van Dyke, 2015). For example, PD has been associated with the progression of atherosclerosis, as human studies show that subjects with PD present thicker carotidal wall (Beck et al., 2001).

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http://dx.doi.org/10.1016/j.archoralbio.2015.11.022
0003-9969/

The mechanisms by which PD can exert systemic effects can be due to both bacterial invasion of remote tissues (direct action) and inflammatory mediators produced in the oral cavity in response to the infection and then released into the circulation (indirect action) (El Kholy et al., 2015). In fact, relevant bacteria from the periodontal infections have been found in atheroma plaques, such as *Aggregatibacter actinomycetemcomitans*,*Porphyromonas gingivalis*, *Bacteroides forsythus* (*Tannerella forsythia*) and *Prevotella intermedia* (Haraszthy, Zambon, Trevisan, Zeid, & Genco, 2000), and thrombi of patients with acute myocardial infarction (*A. actinomycetemcomitans*, *P. gingivalis*, and *Treponema denticola*) (Ohki et al., 2012). In the same way, patients with PD have an impairment of endothelium-dependent vasodilation (Higashi et al., 2009; Amar et al., 2003), which was successfully normalized after the proper periodontal therapy (Elter et al., 2006).

Hasturk et al. (2015) demonstrated that rabbits with experimental periodontitis under a high cholesterol diet exhibit more aortic plaques than the periodontally healthy controls under he same diet. Moreover, Machado et al. (2014) observed that rats with PD present a reduction of the endothelium-dependent vasodilatation due to diminished bioavailability of nitric oxide (NO) and/or other endothelium-derived relaxing mediators.

There is considerable evidence that some prostanoids, along with NO, play important roles in the regulation of vascular tone and blood pressure. Prostaglandins, thromboxanes and prostacyclin from both type-1 and -2 cyclooxygenase (COX) isoforms are the major vasoactive eicosanoids (Katusić & Shepherd, 1991), and the balance between platelet-derived thromboxane A_2 and endothelial prostacyclin is an important factor for the maintenance of vascular homeostasis (Sellers & Stallone, 2008). Previous studies have shown an increased COX-2 expression in mesenteric vessels and a transient systemic and vascular inflammation in animals with a 28 days of bilateral mandibular and maxillary ligature-induced periodontitis (Brito et al., 2013; Mendes et al., 2014).

In this way, and considering that in response to inflammatory stimuli (endotoxin of Gram-negative bacteria alone or together with certain cytokines) there is increased production of both NO (from the inducible nitric oxide synthase isoform–iNOS (Moncada, 1992)) and COX-2-derived prostanoids (Offenbacher, Heasman, & Collins, 1993), we decided to investigate the impact of PD on the in vitro vascular reactivity of rat aorta rings, mainly focusing on the participation of NOS and COX isoforms.

2. Material and methods

2.1. Animals

Male adult Wistar rats (180–200 g) were used in the experiments. During the length of the experimental protocol, the rats were kept in polycarbonate cages (5 animals/cage) in a quiet room with controlled temperature (22 ± 1 °C), humidity (65–75%) and a 12 h light–dark cycle, and received standard rat chow and tap water ad libitum . The animal procedures were approved by the local ethics committee (CEUA-ICB) and were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.2. Induction of periodontitis

Sixteen Wistar rats were anesthetized with ketamine (80 mg/kg, i.p.; Francotar, Virbac do Brasil Ind. e Com. Ltda, Brazil) and xylazine (20 mg/kg, i.p.; Kensol, Konig S.A., Brazil) and divided into 2 groups: Periodontitis (P) and sham (S). The P group ($n = 8$) received a 3–0 cotton ligature in a submarginal position on the lower right first molar to induce periodontitis, as previously described (Sallay et al., 1982; Herrera et al., 2015). Sham-operated animals ($n = 8$) had the ligature immediately removed after the procedure. After 7 days, the mandibles were removed and very carefully dissected in order to maintain their integrity. No adverse events were observed and all P animals kept the ligature until the end of the experimental period.

The mandibles were immersed in 30% hydrogen peroxide for 7 h in order to facilitate the mechanical removal of the soft tissue, and then treated with a 1% methylene blue solution for 25 min for staining of the cemento–enamel junction (CEJ) (Souza et al., 2010).

The mandibles were scanned at 1200 dpi and the images were analyzed blindly by an investigator unaware of the experimental groups, using the software ImageJ (version 1.47; NIH, USA). Alveolar bone loss was estimated by measuring the distance between the CEJ and the alveolar bone crest (ABC) of each root surface for the three molars separately, taking 3 measures for the first molar, 2 for the second and third molars and totalling the bone losses of each root, as previously described (Crawford, Taubman, & Smith, 1978).

2.3. Measurement of systolic blood pressure (BP)

Systolic blood pressure was recorded in the conscious rats before the induction of periodontitis and 7 days after (before the sacrifice of the animals) by the indirect method of tail cuff plethysmography, as previously described (Muscará et al., 1998).

2.4. Preparation of isolated aortic rings

After euthanasia, the thoracic aorta was removed, cleaned off the surrounding fat tissue and constantly kept in aerated (95% O_2, 5% CO_2) Krebs–Henseleit solution at 37 °C. Intact segments (4 mm) of the dissected vessel were mounted in tissue baths for measurement of isometric contractile force (ADInstruments Pty Ltd., Castle Hill, Australia), as previously described (Carvalho, Scivoletto, Fortes, Nigro, & Cordellini, 1987). After the aorta removal, the rings were immediately mounted under a 1.5 g resting tension and, after a 60-min equilibration period, vascular integrity was verified by the contractile response to 84 mM KCl. The vessels were then rinsed with fresh Krebs–Henseleit solution (four times, 15 min each) and the tension was adjusted to 1.5 g. The contractile response to norepinephrine (NE; 10^{-10} to 3×10^{-5} M) was recorded in rings with (E+) and without (E−) endothelium. Endothelium-dependent relaxation was assessed in pre-contracted rings (with 10^{-4} M NE) by the cumulative addition of acetylcholine (ACh, 10^{-10} to 10^{-5} M). The responses to both NE and Ach were also studied in the presence of indomethacin (a non-selective COX inhibitor; 10 μM), SC-560 (a selective COX-1 inhibitor; 9 nM), NS-398 (a selective COX-2 inhibitor; 1 μM), L-NAME (a non-selective NOS inhibitor; 100 μM) or 1400W (a selective iNOS inhibitior; 10 μM), which were added to the bath preparation 15 min before performing the concentration-response curves.

For both NE and ACh, the individual log-transformed concentration vs. response curves were plotted. Maximal response (E_{max}) and potency (as $pD_2 = -\log EC_{50}$, being EC_{50} the drug concentration necessary to cause 50% of the maximal response) values were calculated using the software GraphPad Prism (version 5.01; GraphPad Software Inc., USA).

2.5. Western blot analysis of COX-2

Protein COX-2 expression was analyzed in the aorta tissue samples as previously described (Muscará et al., 2000). Briefly, aorta samples were homogenized and centrifuged (14.000 × g, 2 min). The supernatants (20 μg protein) were subjected to 10% SDS–PAGE electrophoresis. The protein bands were further electro-transferred to nitrocellulose membrane and analysed for the

presence of COX-2 by Western blot. A monoclonal mouse IgG1 anti-rat COX-2 (Transduction Laboratories; diluted 1:250) was used as the primary antibody, and a polyclonal goat anti-mouse IgG coupled to horseradish peroxidase (Jackson Laboratories; diluted 1:6000) as the secondary antibody. Immunoreactive bands were detected by chemiluminescence (ECL kit, Amersham, U.K.).

2.6. Analysis of gene expression by real time-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the aorta samples with TRIzol (Life Technologies, USA) following the manufacturer's instructions. The cDNA was synthesized from 10 μg of total RNA using Superscript II (Life Technologies, GIBCO-BRL, Gaithersburg, MD, USA) and stored at −20 °C until use. RT-PCR was performed using Sybr green to quantify the expression of each isoform of COX, constitutive and inducible isoforms of NOS. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an internal control. All primers showed efficiency higher than 90%. The results were analyzed as $2^{-\Delta\Delta Ct}$. The specific sequences of the primers were: COX-1: CGTGTGTGTGACTTGCTGAA (forward) and GGTTGCGATACTGGAACTGG (reverse), COX-2: ACATTCCCTTCCTTCGGAAT (forward) and AAGGGCCCTGGTGTAG-TAGG (reverse), eNOS: AGCATGAGGCCTTGGTATTG (forward) and CCCGACATTTCCATCAGC (reverse), iNOS: AAAATGGTTTCCCC-CAGTTC (forward) and GTGGATGGAGTCACATGCAG (reverse), HPRT: TATGCCGAGGATTTGGAAAA (forward) and ACAGAGGGCCA-CAATGTGAT (reverse),

2.7. Detection of periodontopathogens by PCR

In order to assess the occurrence of bacterial translocation from the oral cavity, samples of gingiva, ligature and aorta were collected under sterile conditions from additional sets of animals 7 days after ligature placement or the simulated procedure ($n = 5$). The samples were manually homogenized and further incubated in a SDS/proteinase K solution at 37 °C during 2 h. Total DNA was extracted by phenol–chloroform method and precipitated with isopropanol.

PCR amplification of bacterial 16S rRNA was performed in a DNA thermocycler, using the primers D88 (5′-AGAGTTTGATYM TGGCTCAG-3′) and E94 (5′-5GAAGGAGGTGWTCC ARCCGCA-3′) (Paster et al., 2002). The PCR products were purified using the QIAquick gel extraction kit (Qiagen, Dusseldorf, Germany) and the 1.65 kbp fragments were revealed after electrophoresis in 1% agarose gel.

2.8. Statistical analysis

The results were expressed as mean ± SEM. Differences among the groups were analyzed by Student *t*-test for unpaired data. Values of $P < 0.05$ were considered as statistically significant.

3. Results

As expected, rats with ligature showed significant alveolar bone loss after 7 days when compared to the sham group, as measured at the first molar (2.09 ± 0.07 vs. 2.96 ± 0.17 mm, $P < 0.001$), second molar (0.71 ± 0.04 vs. 0.98 ± 0.05, $P < 0.001$) or third molar (0.63 ± 0.04 vs. 0.78 ± 0.04, $P < 0.05$; Fig. 1).

No significant differences were observed between the groups in terms of systolic blood pressure 7 days after ligature (Sham: 122.6 ± 1.2 vs. PD: 120.4 ± 1 mmHg).

However, and as shown by Fig. 2, in comparison with the Sham control group, aorta rings from animals with periodontitis showed lower maximal responses in terms of both NE-mediated contraction (2.2 ± 0.1 vs. 2.7 ± 0.1 g, $P < 0.05$; panel A) and ACh-mediated dilatation (43.5 ± 2.6 vs. 71.9 ± 2.2%, $P < 0.001$; panel B). After the mechanical removal of the endothelial layer, NE contracted the vessels from both groups to similar tension values (3.1 ± 0.1 vs.

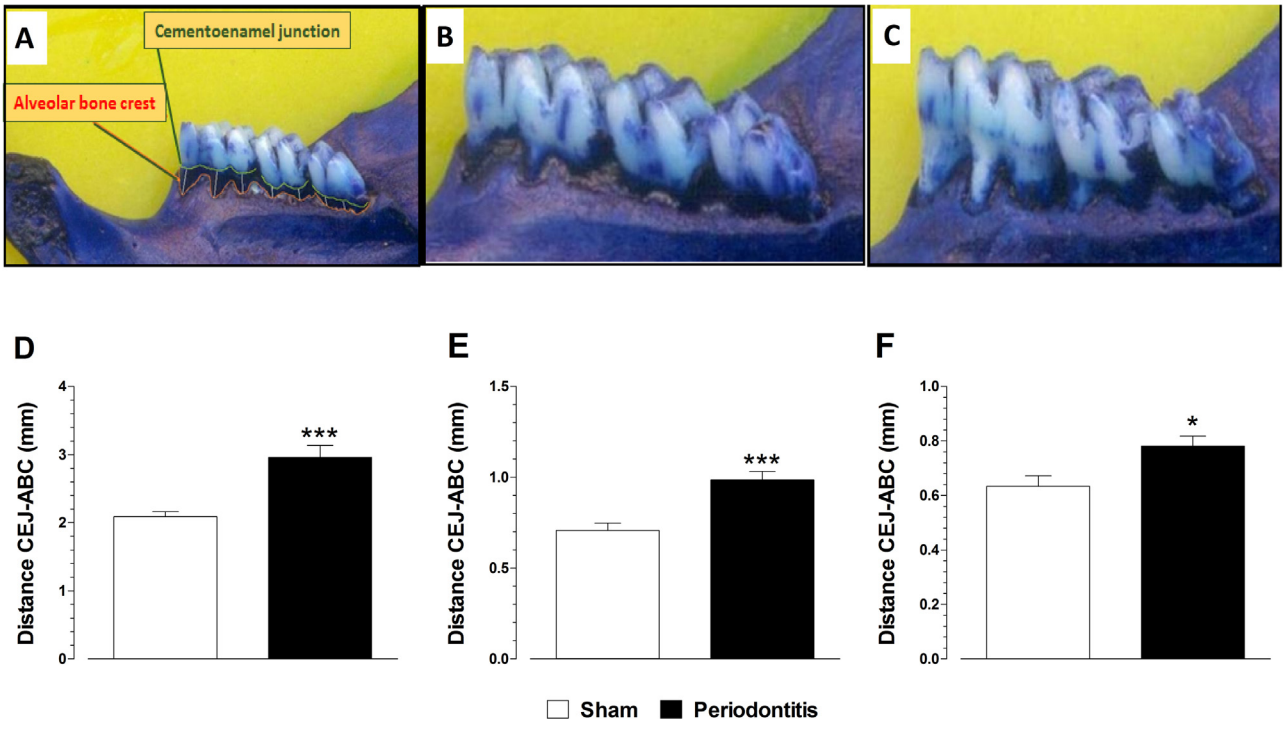

Fig. 1. Scheme showing the measurement of the distances between the cemento enamel junction (CEJ) and the alveolar bone crests (ABC) in methylene blue stained rat mandibles (panel A). Representative mandible images obtained from control animals (Sham; panel B) or seven days after ligature-induced periodontitis (panel C). Bone loss analysis, as measured at the first, second and third molars (panels D, E and F, respectively). $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. Sham ($n = 8$).

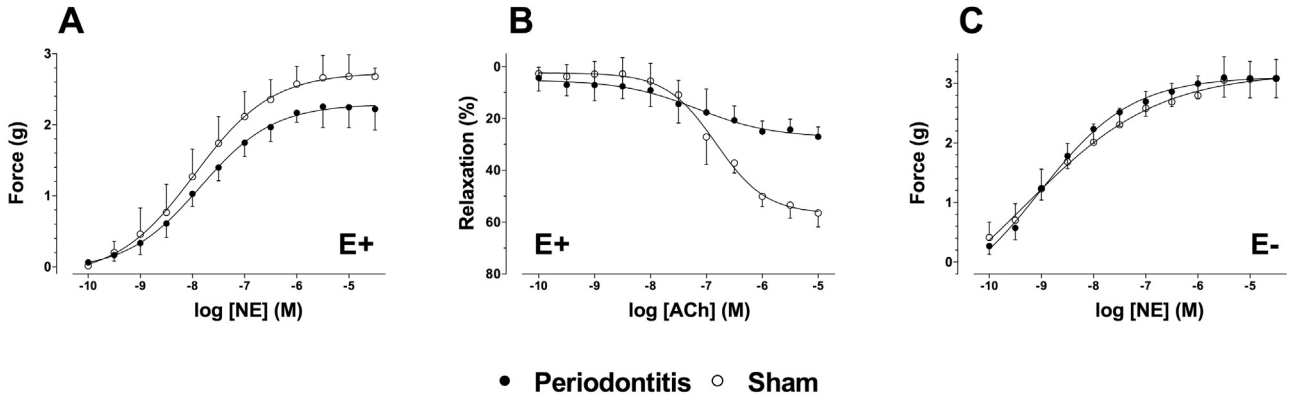

Fig. 2. Effects of unilateral ligature-induced periodontitis on the concentration-response curves obtained for norepinephrine (NE)-stimulated contraction and acetylcholine (ACh)-stimulated relaxation of aorta rings with intact endothelium (E+; panels A and B, respectively). Effect of the mechanical endothelium removal on norepinephrine-stimulated contraction (E−; panel C).

3.1 ± 0.3; panel C), and the relaxing activity of ACh was abolished (not shown).

Fig. 3 shows that the vasomotor differences between the experimental groups were also abolished in the presence of the non-selective NOS inhibitor L-NAME, which significantly increased NE-mediated aorta contraction in both experimental groups to non-statistically significant E_{max} values (PD: 2.9 ± 0.2 g vs. Sham: 2.6 ± 0.1 g; panel A), and almost abolished ACh-mediated dilatation (panel B). In the presence of the selective iNOS inhibitor 1400W, the contractile responses to NE were not statistically different between the two experimental groups (panel C). However, despite the similar maximal relaxant responses to ACh showed by the vessels in the presence of 1400W, ACh potency was significantly increased in the aorta rings from rats with periodontitis, as assessed by the calculated pA_2 values (PD: 7.35 ± 0.14 vs. Sham: 6.65 ± 0.19, $P < 0.05$; panel D).

The maximal responses to both NE and ACh were also absent when indomethacin or NS-398 (a non-selective COX and a selective COX-2 inhibitor, respectively) were added to the tissue bath (Fig. 4, panels A, B, D and E). However, in comparison with the Sham

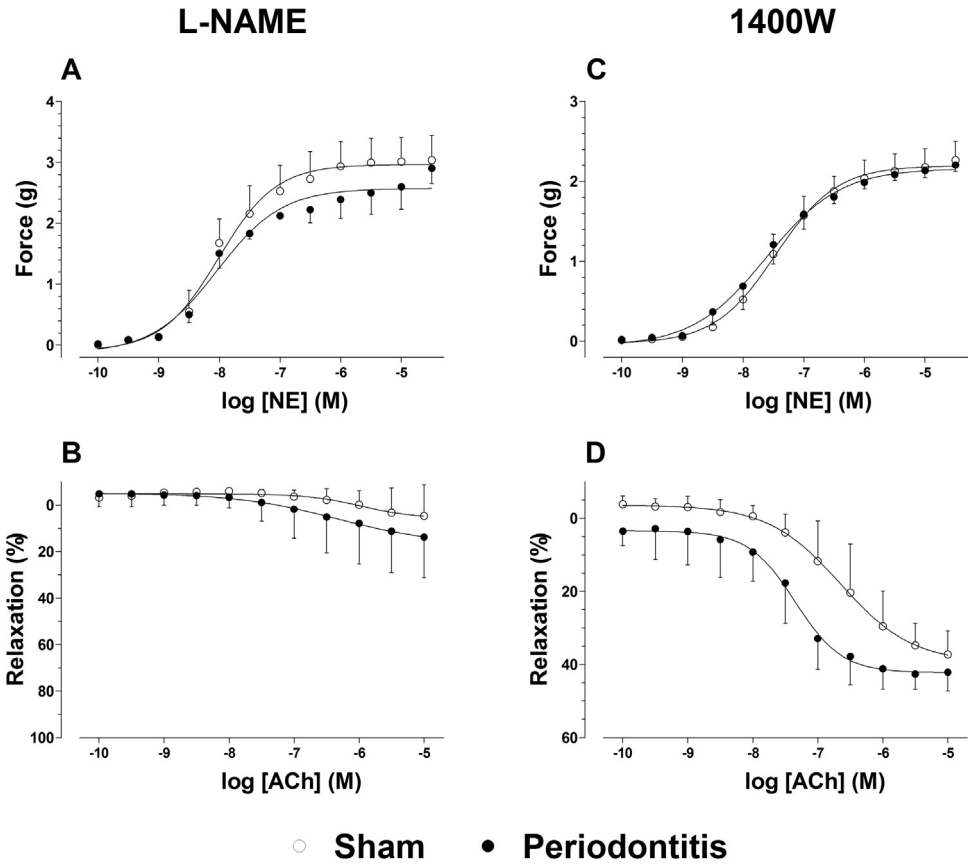

Fig. 3. Effects of unilateral ligature-induced periodontitis on the concentration-response curves obtained for norepinephrine (NE)-stimulated contraction and acetylcholine (ACh)-stimulated relaxation in the presence of the non-selective NOS inhibitor L-NAME (panels A and B, respectively) or the selective iNOS inhibitor 1400W (panels C and D, respectively).

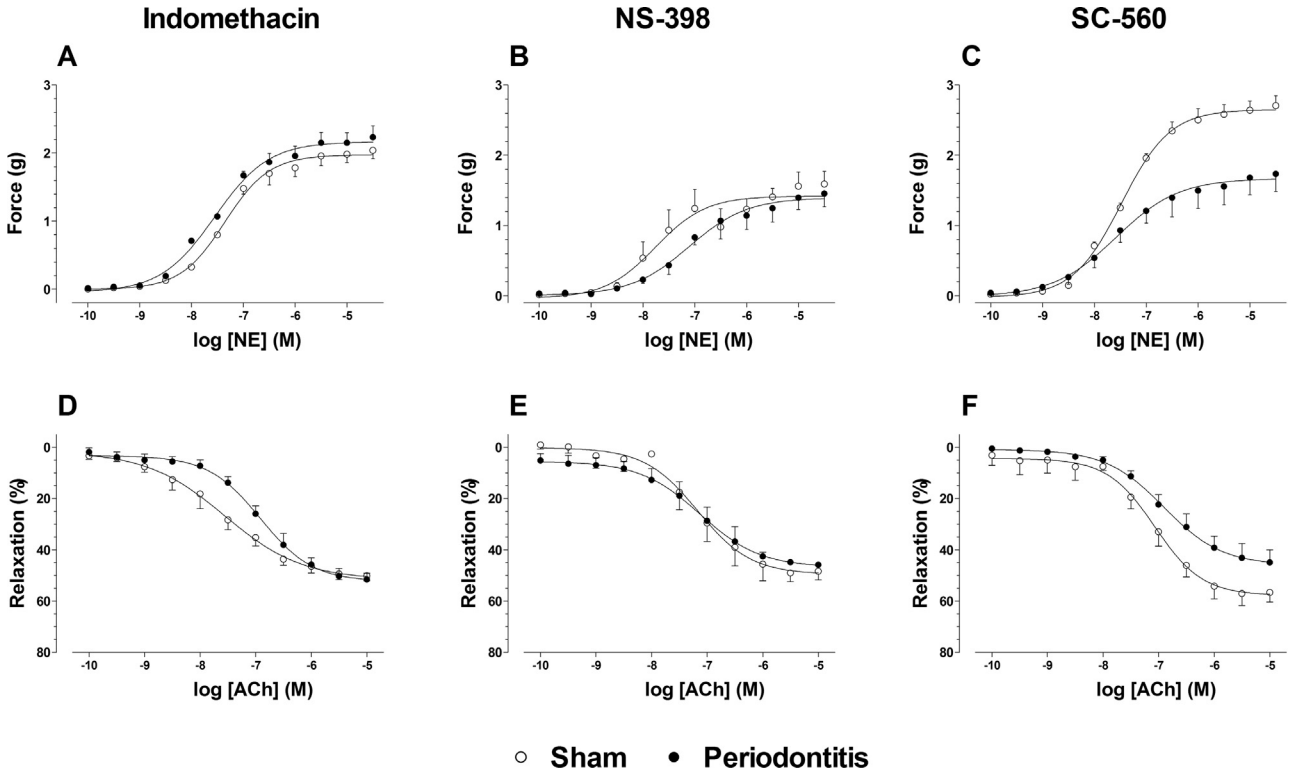

Fig. 4. Effects of unilateral ligature-induced periodontitis on the concentration-response curves obtained for norepinephrine (NE)-stimulated contraction and acetylcholine (ACh)-stimulated relaxation in the presence of the non-selective COX inhibitor indomethacin (panels A and D, respectively), the selective COX-2 inhibitor NS-398 (panels B and E, respectively) or the selective COX-1 inhibitor SC-560 (panels C and F, respectively).

group, the vessels from rats with periodontitis showed lower pA_2 values for ACh in the presence of indomethacin (PD: 6.91 ± 0.09 vs. Sham: 7.56 ± 0.14, $P < 0.05$; panel D) and for NE in the presence of NS-398 (PD: 7.11 ± 0.14 vs. Sham: 7.76 ± 0.20, $P < 0.05$; panel B). In the presence of the selective COX-1 inhibitor SC-560, the vasomotor differences between the groups were unaltered in terms of their responses to both NE and ACh (Fig. 4, panels C and F, respectively).

As shown in Fig. 5, COX-2 expression was significantly increased in the aorta samples from animals with PD in comparison with the Sham group, both in terms of protein (39.7 ± 2.8 vs. 15.6 ± 0.8 A.U., $P < 0.01$, panel A) and mRNA expression (3.1 ± 0.9 vs. 1.0, $P < 0.05$; panel B. No differences between the groups were observed in COX-1 mRNA expression (0.9 ± 0.2 vs. 1.0; panel C). However, mRNA expression was significantly increased for iNOS (2.3 ± 0.4 vs.1.0, $P < 0.05$; panel D) and decreased for eNOS (0.15 ± 0.02 vs. 1.0,

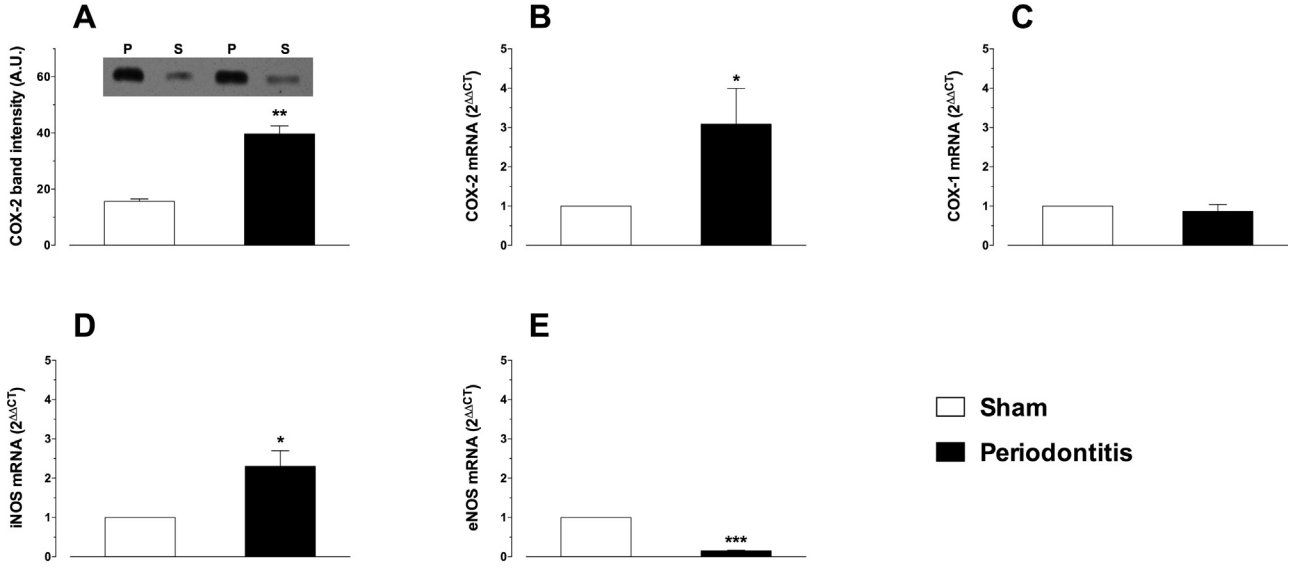

Fig. 5. Protein expression of COX-2 (panel A) and mRNA expression of COX-2 (panel B), COX-1 (panel C), iNOS (panel D) and eNOS (panel E) analysed in aorta samples obtained from control rats (Sham) or seven days after ligature-induced periodontitis. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham ($n = 8$ for each experiment).

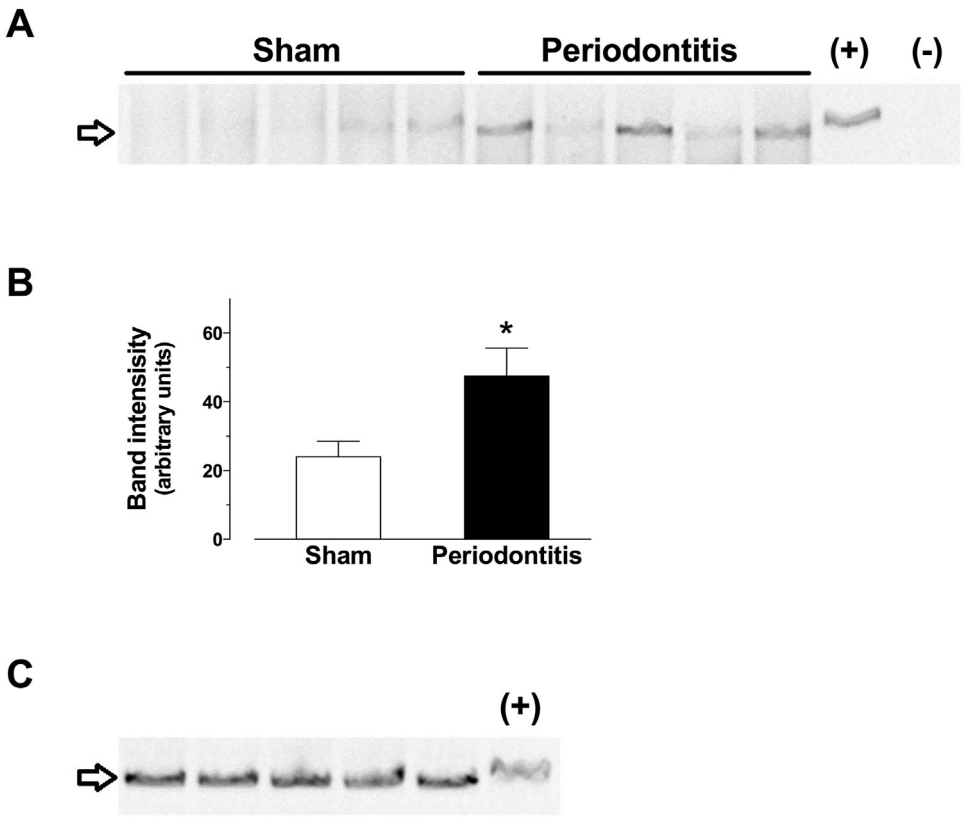

Fig. 6. Bacterial 16S rRNA gene expression analysed in gingiva samples (panel A) collected from rats with experimental PD and densitometric analysis (panel B) of the PCR product. Panel C shows that ligatures collected from rats with PD show the same bacterial PCR product. The arrows show the PCR product corresponding to 1.65 kbp; (+): positive control (5 ng of genomic *E. coli* DNA); (−): negative control (no DNA); $^{*}P < 0.05$ vs Sham ($n = 5$ for each group).

$P < 0.05$; panel E) in the aorta samples obtained from the animals with periodontitis in comparison with the Sham control group.

PCR analysis of bacterial 16S rRNA showed no signal detection in the collected aorta samples from either experimental group, despite all the ligature and gingiva samples collected from rats with periodontitis were positive for this marker (Fig. 6).

4. Discussion

The association between oral diseases and general health has attracted attention over the last three decades, and the World Health Organization has acknowledged the importance of oral health care in the prevention of fatal chronic diseases (Petersen & Yamamoto, 2005). For example, the number of missing teeth, due to current or past periodontitis, may indicate an increased risk for CVD, diabetes mellitus, and all-cause mortality, thus making of the degree of edentulism an additional risk factor that could be added to the existing cardiovascular risk profiles (Liljestrand et al., 2015).

In this study, we show that, in the presence of unilateral periodontitis (as confirmed by the significant alveolar bone loss present in the animals), the in vitro vascular response of aorta is impaired in terms of both adrenergic contraction and endothelium-dependent relaxation. In addition, considering that the mechanical removal of the endothelial layer overcomes these differences between the groups, these results confirm that the presence of ligature-induced periodontitis in rats leads to the development of endothelial dysfunction. Furthermore, the pharmacological results obtained in the presence of selective and non-selective inhibitors for the different NOS and COX isoenzymes, clearly show that iNOS-derived NO and COX-2-derived prostanoids must be among the endothelial factors that mediate the mentioned dysfunction. The findings on increased mRNA expression for iNOS and COX-2 (as well as COX-2 protein), together with the decreased eNOS mRNA confirm the pharmacological evidences.

Endothelial dysfunction is the first step in the process of atherosclerosis, and the plausible association of periodontal bacteria with the progression of endothelial dysfunction and atherosclerosis has been addressed through three main hypothesis (Gurav, 2014).

The immunological hypothesis supports that the host may harbor a hyper-inflammatory monocyte phenotype which would result in the release of an abnormally high amount of pro-inflammatory mediators when the leukocytes are challenged by the bacterial antigens (Houri-Haddad, Wilensky, & Shapira, 2007; Shaddox et al., 2010; Herrera et al., 2014). In fact, it has been shown that individuals with this phenotype are at a higher risk of developing periodontitis and endothelial dysfunction (Gurav, 2014).

The inflammatory theory is based on the fact that, in response to the presence of periodontopathogens, gingival cells can produce inflammatory mediators that are released into the systemic circulation, which can, in turn, affect endothelial function. In fact, the results shown in this study support this hypothesis, since the presence of PD leads to increased aortic expression of iNOS and COX-2 and reduced eNOS.

We have previously shown that NO has a key role in the course of ligature-induced periodontitis in rats, as treatment of the animals with either L-NAME or aminoguanidine (an iNOS inhibitor) results in diminished bone loss secondary to inhibition of osteoclast differentiation and activity (Herrera et al., 2011a,b).

Despite the deleterious local and remote (e.g. cardiac and renal) effects of iNOS-derived NO and other pro-oxidative derivatives (such as peroxynitrite anion) (Herrera, Martins-Porto, Campi, et al., 2011; Herrera, Martins-Porto, Maia-Dantas, et al., 2011), these species are part of the host defense mechanisms against microbes (Gyurko et al., 2003), thus confirming the "friend-and-foe" characteristics of NO.

According to the bacteriological hypothesis, periodontal pathogens may penetrate and colonize the vascular endothelial cells, thus contributing to the inflammatory process (Li, Michel, Cohen, Decarlo, & Kozarov, 2008; Roth et al., 2007; Kozarov, Dorn, Shelburne, Dunn, & Progulske-Fox, 2005). It is worth while mentioning that the total ulcerated area in the periodontal pockets present in individuals with severe periodontitis is equivalent to 15–20 cm^2 (Loos, 2005), thus providing periodontal bacteria (and their products) an easy access to systemic circulation.

Although the nature of the association between PD and CVD is still a matter of debate, live periodontal pathogens effectively can reach systemic vascular beds. Indeed, animal studies show that PD bacteria can promote atherosclerosis and that non-invasive mutants cause significantly less severe lesions (El Kholy et al., 2015; Reyes, Herrera, Kozarov, Roldá, & Progulske-Fox, 2013). Nevertheless, there are some questions that still remain to be answered, as for example those related to the relationship between the inflammatory response and the phenotype and pathogenicity of the bacteria deposited in atherosclerotic plaques, the consequences of periodontal treatment on primary myocardial infarction ocurrences and the relevance of bacteria phenotype in the bacteria- endothelium interaction, among others (El Kholy et al., 2015).

Using the model of ligature-induced periodontitis in rats, Theodoro, Pires, Fernandes, Longo, & de Almeida Juliano (2015) have already observed that the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans* are present in the ligature 7 days after the PD induction. Moreover, Duarte, Tezolin, Figueiredo, Feres, & Bastos (2010), using checkerboard DNA–DNA hybridization for human periodontal species, showed that twenty five species of bacteria were found in the ligatures, including *P. gingivalis*- and *A. actinomycetemcomitans*-like species after 42 days of PD. *P. gingivalis*, has been shown to induce iNOS expression in gingival fibroblasts, inflammatory cells and basal keratinocytes (Kendall, Haase, Li, Xiao, & Bartold, 2000). It also stimulates the release of iNOS-derived NO from macrophages (Shapira, Champagne, Van Dyke, & Amar, 1998) and induces the expression of interferon-γ, an important stimulus for iNOS expression (Ogawa, Ohno, Kameoka, Yabe, & Sudo, 1994). It is thus feasible that *P. gingivalis* can stimulate NO production either locally or distantly from the periodontium, e.g. the systemic vasculature, as this bacteria has been found in atheroma plaques (Haraszthy et al., 2000) and thrombi from patients with acute myocardial infarction (Ohki et al., 2012). In addition, and strengthening the validity of the animal model used in this study, increased nitration of cardiac proteins (Ma, Guo, You, Xia, & Yan, 2011) and enhanced lipid deposits in aorta (Bain et al., 2009) have been detected in rats seven days after ligature induced-periodontitis.

Our PCR results show that all the ligature and gingiva samples collected at day 7 after PD induction were positive for the gene encoding bacterial 16S rRNA, although no detectable signal was observed in the aorta samples. Based on these results, we can thus suggest that the vascular inflammatory process that leads to endothelial dysfunction is more probably related to inflammatory mediators produced in response to the bacteria present in the oral cavity than a direct interaction of the aorta with periodontopathogens. However, we can not rule out this possibility in other vascular beds (for example, small-caliber resistance vessels).

In some situations, COX-2-derived prostacyclin (PGI_2) may compensate for diminished eNOS functionality. For example, Gödecke et al. (1998) and Sun et al. (2006) showed that in eNOS-deficient mice, COX-2-derived prostanoids compensate for the lack of NO in the regulation of coronary artery hemodynamics and flow-induced dilatation in vivo . In arthritic patients with concomitant vascular disease characterized by decreased NO bioavailability, PGI_2 production and activity is several folds higher than in control patients (Hishinuma et al., 2001; Anning et al., 2006; FitzGerald, Smith, Pedersen, & Brash, 1984); since vascular relaxation is highly dependent on PGI_2 in these patients, treatment with nonsteroidal anti-inflammatory drugs put these patients at higher cardiovascular risks (Anning et al., 2006).

However, this does not seem to be the case in our study as, despite the increased COX-2 expression, aorta rings prepared from the animals with periodontitis show impaired endothelial-dependent relaxation. In fact, this response is highly dependent on NO (as evidenced by the abolishing effects of L-NAME) and more probably related to eNOS downregulation (as evidenced by the RT-PCR experiments).

As a whole, and considering that in the presence of either 1400W or NS398 the aorta rings from both experimental groups exhibited similar maximal vasomotor responses, it is clear that endothelial iNOS-derived NO and COX-2-derived prostanoids are, at least partly, responsible for the altered α_1-mediated vasoconstriction and the induced desensitization/tolerance to eNOS-derived NO.

We have previously shown that COX-2-derived prostanoids have an important role in periodontal disease, as treatment of rats with etoricoxib, a specific COX-2 inhibitor, results in decreased alveolar bone loss secondary to ligature-induced periodontitis (Holzhausen, Spolidorio, Muscará, Hebling, & Spolidorio, 2005). Using the same model, although placing four ligatures instead of one, Brito et al. (2013) have shown that on the 28th day after the induction of periodontitis, thee is a transient systemic and vascular inflammation that leads to impaired endothelium-dependent vasodilatation, and that on the 21st day, increased COX-2 expression ocurs in mesenteric vessels, which endothelial function is worsened by treatment with etoricoxib (Mendes et al., 2014). In this way, the present study not only confirms previous observations but also strengthens the important relationship that exists between PD and endothelial dysfunction, as differently from the long-lasting, more aggressive and generalized periodontal inflammation studied by the above mentioned authors, in our model, data were collected just 7 days after placement of a single ligature.

Our results suggest that there is no involvement of COX-1 when vascular reactivity is evaluated in vitrohowever, the participation of platelet COX-1 derived TxA_2 cannot be ruled out in vivo . In fact, it has been early demonstrated that not only local TxA_2 production is increased during gingivitis and periodontitis (Rifkin & Tai, 1981), but also that aggregation of circulating platelets from patients with periodontitis is enhanced (Krause et al., 1990), which can be, at least partly, due to direct effects of periodontopathogens (Herzberg & Meyer, 1996), thus exacerbating the risks of cardiovascular diseases

As a whole, our results show that significant alterations in aorta vasomotricity ocur in rats with experimental periodontitis, and that the endothelium-derived NO and COX-2 products are involved in these alterations, thus evidencing potential clinical checkpoints for pharmacological intervention.

Conflict of interest

The authors have stated that they have no conflict of interest.

Ethical approval

The animal procedures were approved by the local ethics commitee (CEUA-ICB) and were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Funding

This study has been supported by the State of São Paulo Research Foundation (FAPESP).

Acknowledgements

MNM, MHCC, SKPC and LCS are recipients of fellowships from the Brazilian National Council for Scientific and Technological Development (CNPq). The authors also aknowledge Dr. Rodrigo Martins-Porto, Mrs. Irene Gouveia and Mrs. Marta Rodrigues da Silva for the technical assistance.

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