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

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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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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