Multiple Apical Periodontitis Influences Serum Levels of Cytokines and Nitric Oxide

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Abstract

Introduction: This study evaluated whether apical periodontitis (AP) in a single tooth or in multiple teeth affected serum levels of inflammatory mediators and influenced blood homeostasis. **Methods:** Thirty male Wistar rats were divided into 3 groups of 10 rats each: control group, healthy rats; 1AP group, rats with AP in 1 tooth; and 4AP group, rats with AP in 4 teeth. After 30 days, the rats were anesthetized, and their blood was collected through cardiac puncture to quantify tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-6, IL-17, IL-23, and nitric oxide (NO) levels. The rats were then sacrificed by administering an anesthetic overdose. Their maxillary and mandibular molars were collected and processed for histologic analysis with hematoxylin-eosin and for immunohistochemical staining of the cytokines and NO-producing enzyme nitric oxide synthase. Results of these analyses were statistically analyzed; $P < .05$ was considered statistically significant. **Results:** Rats in the 1AP and 4AP groups showed increased IL-6, IL-17, IL-23, TNF-α, IFN-γ, and NO synthase expression; inflammatory cell infiltration; and moderate bone resorption in affected teeth. Serum TNF-α, IL-6, IL-17, and IL-23 levels were higher in rats in the 4AP group than in those in the control group ($P < .05$). Serum NO levels were significantly lower in rats in the 1AP and 4AP groups than in those in the control group ($P < .05$). Serum IFN-γ levels were not different among rats in the 3 groups ($P > .05$). **Conclusions:** These results suggested that AP affected blood homeostasis by altering the serum levels of inflammatory cytokines and NO. *(J Endod 2016;42:747–751)*

Key Words

Apical periodontitis, cytokines, nitric oxide

The theory of focal infection, which was proposed in the early 20th century, suggests that pathogenic microorganisms causing dental infections can disseminate systemically and trigger systemic changes (1). Thus, the prescribed treatment for infected teeth was extraction (2). This theory was rejected because of the lack of scientific evidence (3). However, results of several recent studies on this topic indicate that dental infections such as apical periodontitis (AP) may potentiate the pathogenesis of autoimmune diseases, mainly by increasing inflammatory cell infiltration and serum cytokine levels (4–8).

The pathogenesis of AP is complex and involves inflammatory mediators that activate as well as inhibit inflammation (9). Pro- and antiinflammatory cytokines are produced by T-helper (Th) cells seeking to neutralize the pathogenic agents (10). Cells belonging to the Th1 lineage enhance the inflammatory process by secreting cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which activate osteoclastogenesis and polymorphonuclear cells (11, 12). Cells belonging to the recently discovered Th17 lineage release cytokines such as interleukin (IL)-17 and IL-23 (13). Besides, in AP, Th17 cells and Th1 cells, which are also associated with increased bone resorption, are considered proinflammatory (14). IL-6 is produced by several cell types and is involved in acute-phase responses, B-cell maturation, and macrophage differentiation. In addition, IL-6 plays a dual role in Th1/Th2 differentiation (15).

Nitric oxide (NO) is an important inflammatory mediator involved in AP (16). NO is a ubiquitous free radical produced in various cells by a family of enzymes collectively known as NO synthases (NOSs) (17). Although the role of NO in AP is unknown, studies have shown that NO modulates the levels of proinflammatory cytokines such as IFN-γ and TNF-α during the pathogenesis of AP (18, 19).

Our previous research showed that periodontal diseases induce systemic alterations. Although periodontal diseases or AP in a single tooth do not induce significant systemic changes, periodontal diseases or AP in 2 teeth alter some systemic parameters (5–7). Therefore, the present study evaluated the serum levels of TNF-α, IFN-γ, IL-6, IL-17, IL-23, and NO in rats with AP in a single tooth or in multiple teeth.

Materials and Methods

Experimental Design

Three-month-old male Wistar rats (weight, 250–280 g) were housed in a temperature-controlled room (25°C) with a 12-hour/12-hour dark/light cycle. The rats had *ad libitum* access to food and water. Experimental protocols of this study

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were approved by the institutional ethics committee and were conducted in accordance with relevant guidelines (Ethics Committee on Animal Use, Universidade Estadual Paulista 2014-00108). The rats were divided into 3 groups of 10 rats each: control group, rats without AP; 1AP group, rats with AP in 1 tooth; and 4AP group, rats with AP in 4 teeth.

Induction of Oral Infections

Rats in the experimental groups were anesthetized by administering an intramuscular injection containing 13 mg/kg xylazine (Coopazine; Coopers Ltd Brazil, São Paulo, Brazil) and 87 mg/kg ketamine (Vetaset; Fort Dodge Animal Health Ltd, São Paulo, Brazil). AP was induced by exposing the pulp on the mesial surface of the first right maxillary molar of rats in the 1AP group and the first and second right maxillary and mandibular molars of rats in the 4AP group by using surgical round burs (Broca LN Long Neck; Maillefer, Dentsply Ind e Com Ltda, Petrópolis, RJ, Brazil). AP was induced by exposing the pulps for 30 days (7, 8).

Assessment of Serum Levels of TNF-α, IFN-γ, IL-6, IL-17, and IL-23

After 30 days, venous blood samples (5 mL) were collected from the anesthetized rats through cardiac puncture after fasting the rats overnight for 8 to 12 hours. The blood samples were centrifuged immediately at 1800*g* and 4°C for 15 minutes to obtain plasma, which was immediately stored at −80°C. Serum cytokine levels were assessed by performing capture enzyme-linked immunosorbent assays (ELISAs) using commercial kits (rat TNF ELISA set BD OptEIA, cat #558535; BD Biosciences, San Diego, CA; rat IFN-gamma DuoSet, cat #DY585; R&D Systems, Inc, Minneapolis, MN; rat IL-6 ELISA set BD OptEIA, cat #550319; BD Biosciences; rat IL-17A ELISA MAX Deluxe, cat #437904; Biolegend, San Diego, CA; rat IL-23 ELISA kit, Uscn Life Science Inc, cat #SEA384Ra; Wuhan, Hubei, China) according to the manufacturers' instructions.

Assessment of Serum NO Levels

Serum NO levels were quantified using the Griess reaction as described by Guevara et al (20). For nitrate reduction, 100-μL serum samples were incubated with nitrate reductase (50 mU/100 μL of the sample), nicotinamide adenine dinucleotide phosphate, and reduced tetrasodium salt hydrate diluted in 20 μmol/L Tris buffer for 30 minutes at room temperature. After reduction, the serum samples were incubated overnight with 900 μL methanol:diethyl ether mixture (3:1 v/v). The samples were then centrifuged (at 10,000 rpm and 4°C for 10 minutes), and the supernatant was used for determining nitrite levels. For this, 50 μL 6.5 mol/L HCl and 50 μL 37.5 μmol/L sulfanilic acid were added to 200 μL deproteinized supernatant samples. After incubation at 4°C for 10 minutes, 50 μL 12.5 mmol/L N-1-naphthylethylenediamine was added, and the samples were incubated for 30 minutes at 4°C. The samples were then centrifuged (at 10,000 rpm and 4°C for 10 minutes), and their absorbance was measured at 540 nm by using a microplate reader (Labsystems, Midland, ON, Canada). A standard curve was generated using sodium nitrite concentrations ranging from 1–100 μmol/L.

Histopathological and Immunohistochemical Analyses

After obtaining blood samples, the rats were sacrificed by administering an overdose of sodium thiopental (150 mg/kg) intraperitoneally. Their maxillary and mandibular molars were immediately dissected, post-fixed in neutral-buffered formalin for 18 hours (for fixing the samples and for preserving the antigenicity and tissues) (21), and decalcified in 17% buffered EDTA (pH = 8; Sigma-Aldrich, St Louis, MO). The first molar was sectioned semiserially (thickness = 4 μm) along its longitudinal axis (21). The sections were stained with hematoxylin-eosin for histologic analysis, or they were used for performing immunohistochemical staining with an indirect immunoperoxidase technique by using primary antibodies against TNF-α, IFN-γ, IL-6, IL-17, IL-23, and NOS (Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemical staining was performed according to a protocol described previously (21).

The intensity of inflammation was graded as follows: no inflammation (score 1: 0 or few inflammatory cells), mild inflammation (score 2: <25 inflammatory cells), moderate inflammation (score 3: 25–125 inflammatory cells), and severe inflammation (score 4: >125 inflammatory cells). Semiquantitative immunolabeling of TNF-α, IFN-γ, IL-6, IL-17, IL-23, and NOS was performed by a certified histologist who was blinded to the treatments. Three histologic sections were used for each animal, and positive immunoreactivity (IR) was defined as a brownish color in the cytoplasm of the cells and extracellular matrix. Because immunolabeling of both the cells and the extracellular matrix is of great importance for our study, we performed semiquantitative analysis, which provides information on the numbers of immunoreactive cells and immunolabeling intensity of the extracellular matrix. The scores were assigned as follows (22): 0, complete absence of immunoreactive cells; 1 (low IR), a few immunoreactive cells and weak labeling of the extracellular matrix (approximately one quarter of the immunoreactive cells); 2 (moderate IR), a moderate number of immunoreactive cells and moderate labeling of the extracellular matrix (approximately one half of the immunoreactive cells); and 3 (high IR), a large number of immunoreactive cells and strong labeling of the extracellular matrix (approximately three quarters of the immunoreactive cells).

Statistical Analysis

Statistical analyses and data tabulation were performed using SigmaPlot software (Systat Software Inc, San Jose, CA). Nonparametric data were analyzed by performing multiple comparisons with the Kruskal-Wallis test followed by the Dunn test. Parametric data were analyzed by performing analysis of variance followed by the Tukey test. The level of significance was set at 5%.

Results

Serum Levels of TNF-α, IFN-γ, IL-6, IL-17, and IL-23

Serum levels of TNF-α, IFN-γ, IL-6, IL-17, and IL-23 are shown in Table 1. Serum levels of TNF-α, IL-6, IL-17, and IL-23 were significantly higher in rats in the 4AP group than in rats in the control group ($P < .05$). Although rats in the 1AP group showed an increase in the serum levels of most cytokines compared with rats in the control group, the difference was not statistically significant ($P > .05$). Serum IFN-γ levels were not different among rats in the 3 groups ($P > .05$). Moreover, no difference was observed between rats in the 1AP and 4AP groups with respect to the levels of TNF-α, IL-6, IL-17, and IL-23 ($P > .05$, Table 1).

Serum Levels of NO

AP decreased serum NO levels in rats ($P < .05$, Table 1). Serum NO levels were significantly lower in rats in the 1AP and 4AP groups than in rats in the control group ($P < .05$). However, no significant difference was observed in serum NO levels between rats in the 1AP and 4AP groups ($P > .05$).