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ORIGINAL ARTICLE

Endodontic infections increase leukocyte and lymphocyte levels in the blood

Renata Oliveira Samuel[1,2] · João Eduardo Gomes-Filho[1] · Mariane Maffei Azuma[1,3] · Dóris Hissako Sumida[4] · Sandra Helena Penha de Oliveira[4] · Fernando Yamamoto Chiba[4] · Suely Regina Mogami Bomfim[5] · Paulo César Ciarlini[5] · Luis Gustavo Narciso[5] · Luciano Tavares Angelo Cintra[1]

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Abstract
Objectives The aim of this study was to determine whether the presence of single or multiple apical periodontitis (AP) alters blood cell counts and cytokine production.
Material and methods Thirty rats were divided into three groups: a control group comprising rats without AP, a group called 1AP comprising rats with AP in one tooth, and a group called 4AP comprising rats with AP in four teeth. Endodontic infection was induced by pulp exposure of the first right maxillary molar in the 1AP group or by exposing the first and second right maxillary and mandibular molars in the 4AP group. A blood count and cytokine levels were obtained 30 days after infection by collecting blood by cardiac puncture. The maxillae were dissected and stained with hematoxylin and eosin to evaluate the inflammatory infiltrate. The data were tabulated and subjected to statistical analysis ($P < 0.05$).
Results Histological analysis showed a predominance of mononuclear inflammatory cells. In blood, significant increase of leukocytes, lymphocytes, and tumor necrosis factor alpha (TNF-α) in 4AP compared with the control and 1AP groups ($P < 0.05$) was observed. In addition, significant decrease of interleukin-4 (IL-4) in 1AP and 4AP groups compared with the control was observed ($P < 0.05$).
Conclusions In the rat model, the presence of multiple AP can affect health by increasing lymphocyte and TNF-α levels in the blood.
Clinical relevance The presence of endodontic infections can interfere with the blood profile, altering systemic health.

Keywords Apical periodontitis · Leukocytes · Lymphocytes · Blood count

✉ Luciano Tavares Angelo Cintra
lucianocintra@foa.unesp.br

[1] Department of Endodontics, School of Dentistry, São Paulo State University (Unesp), R: José Bonifácio, 1193, Araçatuba, São Paulo, Brazil

[2] Department of Clinical Dentistry, Dental School, UNIUBE—Universidade de Uberaba, Uberaba, MG, Brazil

[3] Department of Endodontics, Ingá University Center, UNINGÁ, Rod. PR 317, 6114 - Parque Industrial 200, Maringá, PR 87035-510, Brazil

[4] Department of Basic Sciences, School of Dentistry, São Paulo State University (Unesp), Araçatuba, SP, Brazil

[5] Department of Veterinary Clinical Laboratory, School of Veterinary Medicine, São Paulo State University (Unesp), Araçatuba, SP, Brazil

Introduction

Infection of endodontic origin is very common in the general population. They are primarily characterized by the presence of harmful agents and a complex immune system that functions by (i) the neutralization of bacteria and their products and (ii) the induction of tissue repair [1]. However, the equilibrium of this process depends on the presence of mediators, which activate and inhibit inflammation to avoid deleterious effects [2]. These effects, which are stimulated by high bacterial load [1] or by diseases that affect the immune system, elevate levels of the mediators in the blood and activate inflammation [3, 4].

The increase of inflammatory cells and mediators in the blood potentiates local inflammatory processes, such as apical periodontitis (AP) [5], and may accelerate the pathogenesis of autoimmune diseases such as rheumatoid arthritis and psoriasis [6]. Therefore, it has been suggested that a bidirectional relationship exists between dental infections and systemic health [7, 8].

Previous studies have shown that infections of dental origin, especially periodontal disease, have a direct relationship with autoimmune diseases [3, 4] and potentiate metabolic upsets by

increasing glycosylated hemoglobin [4, 9], triglycerides [10, 11], inflammatory cells, mediators [4], and oxidative stress [12]. However, few studies have been conducted with respect to infection of endodontic origin. It was previously reported that the presence of AP or periodontal disease localized in one tooth led to slight alterations in the blood [3, 4, 13]. However, when periodontal disease was concomitant with AP, significant alterations in blood and organs were observed [13, 14]. To date, there are few evidences that endodontic infection alone can lead to alterations [15, 16]. Thus, it is possible that the presence of more than one tooth with AP may lead to a significant alteration in inflammatory cells and mediators, as observed previously, when pro-inflammatory cytokines and nitric oxide were evaluated [17].

The blood count is a highly reliable test and has been used for blood evaluation in several studies [13, 18, 19]. It is possible to evaluate several blood parameters such as the levels of erythrocytes, leukocytes, neutrophils, and lymphocytes. Those cells are directly related to the presence of infection such as AP [13]. Regarding inflammatory mediators, tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine related with AP progression [20]. On the other hand, interleukin-4 (IL-4) has been described as a potential protective mediator suppressing pro-inflammatory responses [21]. Thus, those cytokines may be related to the imbalance of bone metabolism in the periapical area, as well as to the blood alterations in consequence of AP.

Thus, the aim of this study was to assess blood counts and TNF-α and IL-4 serum levels from rats with single or multiple AP to determine the alterations in the blood profile linked to AP.

Material and methods

Experimental animals

The experimental protocol was approved by the institutional ethics committee and was conducted in accordance with the relevant guidelines of the Ethics Committee on Animal Use (2014-00108). Three-month-old male Wistar rats weighing 250–280 g were housed in a temperature-controlled room (25 °C) with a 12-h dark/light cycle. Food and water were provided ad libitum.

Induction of AP

The rats were divided into three groups of ten rats each: a control group—rats without AP, a group called 1AP—rats with AP on the first molar of the right maxillae, and a group called 4AP—rats with AP on the first and second right maxillary and mandibular molars.

Anesthesia was administered to each rat in the experimental groups by an intraperitoneal injection of xylazine (13 mg/kg; Coopazine; Coopers Ltd. Brazil, São Paulo, Brazil) combined with ketamine (87 mg/kg; Vetaset; Fort Dodge Animal Health Ltd., São Paulo, Brazil). For the induction of AP, the pulps of the molars were exposed on the mesial surface by using a surgical round bur of 0.1 mm diameter (Broca LN Long Neck, Dentsply Maillefer Ind. e Com. Ltda, Petrópolis, Brazil).

Blood sample collection, determination of hematologic parameters, and assessment of serum levels of IL-4 and TNF-α

After 30 days, the rats were again anesthetized as previously described and a cardiac puncture was performed to collect 5 mL blood from each subject. The samples were collected in ethylenediaminetetraacetic acid (EDTA), mixed, and immediately transferred to a technician, who was blinded to the case status, for processing. The following parameters were determined using an automated analyzer (ABX Micros ABC Vet; Horiba ABX Diagnostics, Montpellier, France): red blood cell concentration; packed cell volume (also known as hematocrit); mean corpuscular hemoglobin level; mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC); and leukocyte, neutrophil, lymphocyte, monocyte, basophil, and eosinophil counts.

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 IL-4 PICOKINE™ ELISA kit #EK0406; Boster Biological Technology, Pleasanton, CA), according to the manufacturers' instructions. Next, the values for each of those hematologic parameters were subjected to analysis of variance followed by the Tukey's test ($P < 0.05$). The cytokine levels were subjected to Kruskall–Wallis test with Dunn's posttest ($P < 0.05$).

Tissue processing and morphometric evaluation

After blood sample collection, the rats were sacrificed using an overdose of anesthetic solution. Their maxillae were removed and subjected to conventional histological processing, as described in a previous study [13].

The extent and severity of the inflammation were evaluated by examining the inflammatory infiltrate. The average number of cells per field in the inflammatory infiltrate and the extent of the inflammation beyond either apical foramen were considered. For each experimental group, the number of cells in the infiltrate was calculated as the average of 10 separate fields (400× magnification), according to the method described in a previous study [4]. The severity of the inflammation was evaluated by assigning one of the following grades to the infiltrate: absent (0 to few inflammatory cells: score 1), mild (< 25 cells: score 2), moderate (25–125 cells: score 3), and severe (> 125 cells: score 4). Analyses were performed blindly by a single calibrated operator. Inflammation scores were statistically analyzed (Kruskal–Wallis test, $P < 0.05$) [4].

Table 1 Mean and standard deviation of blood cell parameters

Hematologic parameters	Groups (mean ± SD)*		
	Control group	1AP	4AP
Leukocytes ($10^2/\mu L$)	58.54 ± 0.83[a]	65.62 ± 1.53[a]	79.69 ± 0.81[b]
Lymphocytes ($10^2/\mu L$)	39.39 ± 7.65[a]	43.50 ± 8.39[a]	56.70 ± 9.56[b]
Neutrophils ($10^2/\mu L$)	18.42 ± 4.34[a]	19.35 ± 3.86[a]	20.86 ± 5.31[a]
Hematocrit	45.33 ± 2.92[a]	44.85 ± 2.11[a]	43.30 ± 1.70[a]
Hemoglobin	15.45 ± 0.91[a]	14.95 ± 0.52[a]	14.50 ± 0.27[a]
MCV (fL)	54.49 ± 0.74[a]	53.50 ± 1.76[a]	52.79 ± 1.11[a]

*Different letters indicate significant statistical differences in lines ($P < 0.05$)

Results

Blood count

The results of the blood tests are shown in Table 1. Among the inflammatory cells, a significant increase in leukocytes and lymphocytes was observed in 4AP compared with the control ($P < 0.05$). Lymphocytes and leukocytes increased to 43.9 and 36% in 4AP, respectively. Monocytes and neutrophils also increased to 23 and 13%, respectively, in 4AP compared with the control; however, this difference was not significant. Mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), eosinophils, erythrocytes, and packed cell volume (also known as hematocrit) showed no significant difference ($P > 0.05$) (Table 1).

Serum levels of IL-4 and TNF-α

Serum levels of IL-4 and TNF-α are shown in Table 2. Serum level of TNF-α was higher in the 4AP group compared with 1AP and control groups ($P < 0.05$). In addition, the levels of IL-4 in the 1AP and 4AP groups were lower compared to control group ($P < 0.05$).

Histologic findings

Representative hematoxylin–eosin-stained sections are shown in Fig. 1. Thirty days after AP induction, the pulp showed total necrosis and AP was established in the 1AP and 4AP groups. The AP was exclusively restricted to the periapex region. Moreover, the AP was composed primarily of neutrophils (polymorphonuclears) and mononuclear cells and comprised moderate inflammatory infiltrate in most samples (Table 2). There was no difference between the 1AP and 4AP groups. In the control group, no sample showed inflammatory infiltrates, and all samples received a score of 0 (Table 3).

Discussion

The present study determined whether single or multiple AP leads to blood alterations. Our results confirmed that endodontic infections in multiple teeth can lead to IL-4 level decrease in blood but increase lymphocyte and TNF-α levels. The histologic features also confirmed this relationship: in addition to the increased levels of lymphocytes in the blood, there were increased levels of lymphocytes in response to bacterial infection in AP in the 1AP and 4AP groups.

The prevalence of lymphocytes in AP is explained by the experimental period of 30 days, since at this stage the lesion has the characteristics of chronic inflammation [22]. In a previous study, we observed similar results in rats with periodontal disease associated with AP [13]. Although there was no significant increase in the presence of dental infection in two teeth, there was an increase of 61% in the average number of leukocytes in the group with AP concomitant with periodontal disease compared with those without dental infections [13].

The increased numbers of inflammatory cells resulting from AP may activate and enhance the deleterious effects of autoimmune diseases. They may also activate inflammatory mediators and increase oxidative stress, exacerbating inflammation in different foci throughout the body [23]. Once the biological pathways that activate inflammation and autoimmunity are the same, the presence of AP can increase the deleterious effects of autoimmune diseases, and the pathogenesis of autoimmune disease can also potentiate the effects of AP in a bidirectional relationship [24, 25]. In this context, we found that the presence of multiple APs increased the level of the pro-inflammatory cytokine TNF-α while it decreased the level of the protective mediator IL-4 in rat's blood.

Table 2 Serum levels of IL-4 and TNF-α (in pg/mL)

Cytokines	Groups (mean, min-max values)*		
	Control group	1AP	4AP
TNF-α	44.87 (26.00–47.15)[a]	46.35 (22.48–66.89)[a]	262.65 (115.63–310.89)[b]
IL-4	26.19 (24.27–28.50)[a]	22.26 (18.02–26.44)[b]	22.39 (16.10–25.24)[b]

Control group rats without apical periodontitis, *1AP* rats with apical periodontitis in 1 tooth, *4AP* rats with apical periodontitis in 4 teeth, *IL* interleukin, *TNF-α*, tumor necrosis factor alpha

*Different letters indicate significant statistical differences in lines ($P < 0.05$)

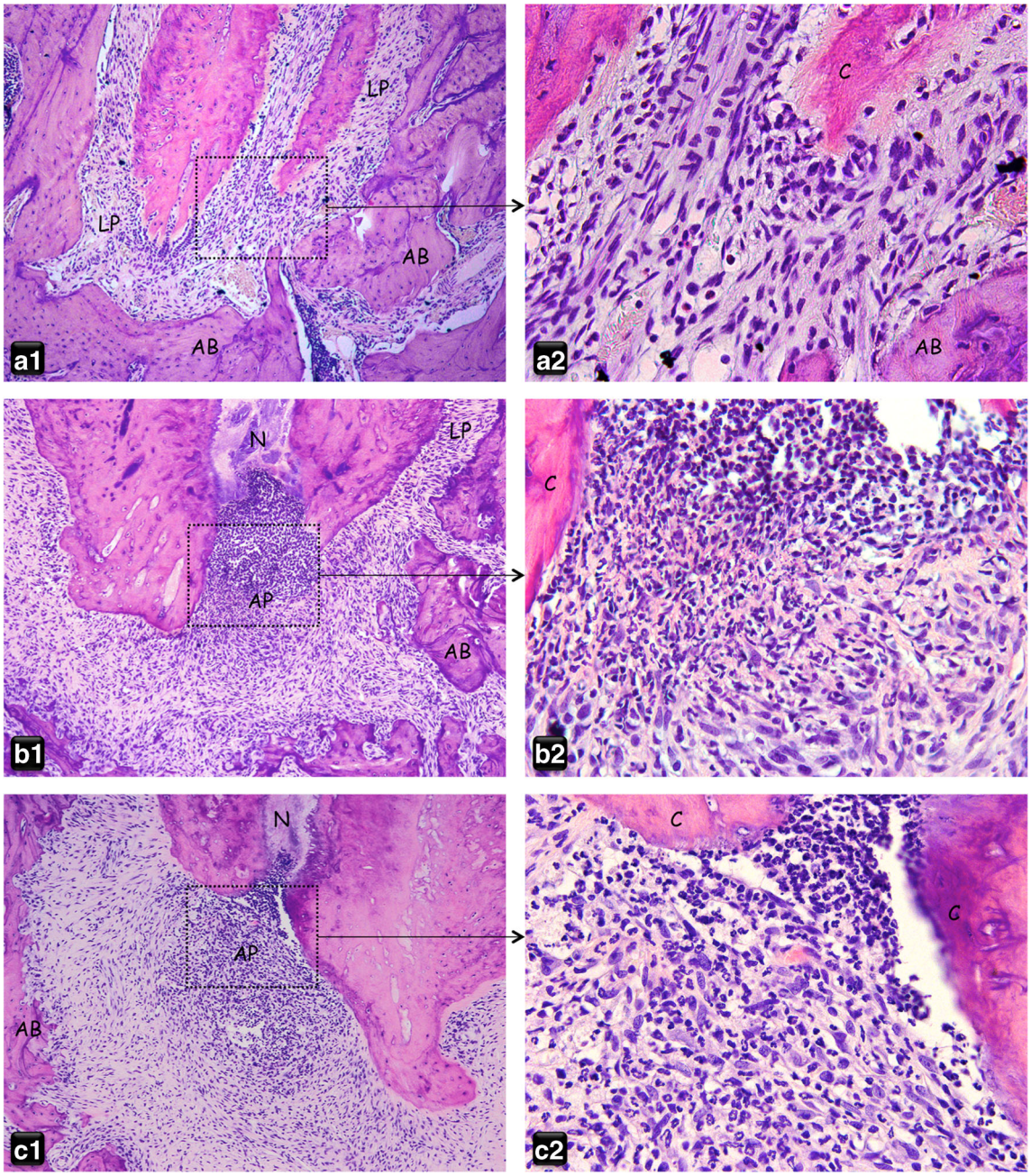

Fig. 1 Histological observations of the control group: (a1,a2). Photomicrographs showing the periapical area with normal features and an absence of inflammatory infiltration at the apical and periapical regions (hematoxylin, 100× and 400×). Representative histological findings 30 days following apical periodontitis (AP) induction: (b1,b2,c1,c2). A sagittal section of a first molar that shows moderate inflammatory cell infiltration near the tooth apex region, severe disorganization of the periodontal ligament, and large areas of bone resorption (hematoxylin, 100× and 400×). PL periodontal ligament, AB alveolar bone, C cementum, N total necrosis, AP apical periodontitis

Serum increase of TNF-α in the presence of AP was demonstrated in another study [17]. Both in AP and in periodontal disease, TNF-α acts in the process of bone resorption, stimulating clastic cells and other mediators that activate inflammation [26]. Moreover, it is known that TNF-α may potentiate the pathogenesis of autoimmune diseases, such as cardiovascular diseases and diabetes [27, 28]. In diabetes mellitus, e.g., insulin resistance was associated with higher level of TNF-α in rats with periodontal disease when compared to diabetic rats without periodontal disease and lower TNF-α level [29].

IL-4 is an immune-regulatory protein secreted by activated Th2 lymphocytes and acts of physiological processes in the organism [30]. Serum and local decreased IL-4 may be harmful. Studies show a greater process of bone resorption in periodontal disease associated with a reduction in IL-4 level [31, 32]. This increase of osteoclastic activity may be due to IL-4 related to the

Table 3 Scores and median of the histological findings

Intensity of inflammatory infiltrate	Groups		
	Control group	1AP	4AP
1—absent	10/10	0/10	0/10
2—mild	0/10	1/10	0/10
3—moderate	0/10	8/10	8/10
4—severe	0/10	1/10	2/10
Median*	1[a]	3[b]	3[b]

*Different letters indicate significant statistical differences in lines ($P < 0.05$)

control of TNF-α release, i.e., the presence of IL-4 can reduce the expression of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α [33]. In the present study, there was decreasing on IL-4 and increasing on TNF-α, as it occurs in some autoimmune diseases, such as cardiovascular disorders [34]. Thus, it is noted that imbalances both pro-inflammatory and anti-inflammatory mediators impair body hemostasis [34].

Confirming this bidirectional relationship, models with diabetes and oral infections, i.e., have shown that various parameters are altered in the presence of systemic diseases and periodontal and periapical lesions. These alterations include increasing on blood glucose [4], glycosylated hemoglobin [4], triglycerides [11], inflammatory mediators such as IL-17 and neutrophils [3], and insulin resistance [27]. Cardiovascular diseases have also been reported to be associated with AP [28]. Moreover, histological and radiographic analysis of the lesions has revealed a significant increase in bone loss in diabetic rats compared with non-diabetic rats with the same dental infections [3].

Our results showed that blood neutrophil levels were not significantly different between the groups. This result is consistent with a previous report, which also showed that neutrophil levels in the presence of concomitant periodontal disease and AP were not significantly different [13]. In diabetic rats with AP and concomitant periodontal disease, the lesions showed acute inflammation even at 30 days, which also led to increased levels of polymorphonuclear cells [13]. This indicates that diabetes enhances the inflammatory process. In the present study, the rats with AP were healthy. Thus, after 30 days, AP showed chronic inflammation, and lower levels of neutrophils between groups were expected, since these cells are the first line of defense [22].

In the present study, there was no significant difference in MCV, hematocrit, and hemoglobin in the presence of AP. However, it was noticeable that all these parameters were slightly reduced in the presence of multiple AP compared with the control group. In the presence of aggressive periodontal disease, some studies have also reported a reduction of red blood cells and hemoglobin levels, suggesting that periodontal disease may be associated with the pathogenesis of mild anemia [35, 36]. Since in the present study AP was induced in only four teeth, the difference between the groups was not significant, as observed in generalized periodontitis.

In the present study, the presence of AP in one tooth did not result in significant differences compared with uninfected rats. These data are consistent with those of a previous study [13] and were therefore expected. However, a slight increase in some parameters suggested the need for induction of infection in multiple teeth to observe significant differences, as observed in some patients. It is common for a single patient to show foci of endodontic infections in multiple teeth [37].

Experimental induction of AP in rats is a consistent method and an accepted model for studying AP [38]. The use of experimental animal model allowed to perform the study under standardized conditions where animals of the same strain, sex, and weight received the same food, experienced the same stress, and shared the same accommodation [38]. Similar study in humans would not be ideal owing to the variability of samples, which could put the results into question. In addition, blood count is an extremely reliable test and has been used in several medical evaluations and scientific studies [13, 18, 19]. For those reasons, we found it prudent to use blood count in rats to confirm the hypothesis that infection of endodontic origin may influence systemic health.

The scientific community has not extensively explored the relationship between AP and systemic health. However, the existence of this relationship in periodontal disease has been well established by several in vivo and in vitro studies [39–41]. Although both infections have a similar pathogenesis with similar bacterial profile and inflammatory process with subsequent bone resorption, the treatment of endodontic infection is still not completely seen as a holistic health promotion.

Given the results of this study, it is noted that AP can activate or enhance the deleterious effects of autoimmune diseases, owing to the significant systemic alterations that occur in the blood, such as increased lymphocyte counts and pro-inflammatory mediators.

Conclusion

In conclusion, the presence of multiple apical periodontitis can affect health by increasing lymphocyte and TNF-α levels and decreasing IL-4 levels in the blood.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants performed by any authors. The animal study was approved by the Institutional Ethics Committee (CEUA 2014-00108) of Universidade Estadual Paulista, São Paulo, Brazil and conducted in accordance with ethical standards.

Informed consent For this type of study, formal consent is not required.

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