

Radiographic and Immunohistochemical Evaluation of Root Canal Treatment Using Different Irrigation Systems

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The aim of this study was to evaluate the radiographic periapical repair and the synthesis of inflammatory mediators after endodontic treatment in a single session, using different irrigation protocols, in teeth with apical periodontitis. Experimental apical periodontitis were induced in dog's teeth randomly assigned into 4 groups: G1 - Irrigation by Negative Apical Pressure (n= 20); G2 - Passive Ultrasonic Irrigation (n= 20), G3 - Positive Pressure Irrigation (n= 20); G4 - apical periodontitis without treatment (n= 20). After 180 days, the animals were euthanized, the tissues removed and submitted to histotechnical processing for immunohistochemical analysis of osteopontin (OPN), tumor necrosis factor- α (TNF- α) and interleukin 1- α (IL-1 α). Radiographic analysis was performed using the Periapical Index (PAI), obtained prior to and 180 days following endodontic treatment. Data were analyzed using Wilcoxon signed-rank test, Fisher's Exact test or Kruskal-Wallis test and Dunn's post-test ($\alpha = 5\%$). Radiographically, after endodontic treatment, apical periodontitis persisted in 35% of G1 specimens, 40% of G2 and 40% of G3 ($p>0.05$), although a PAI reduction was observed ($p<0.05$). By immunohistochemical evaluation, endodontic treatment resulted in lower synthesis of TNF- α and OPN in periapical region, compared to apical periodontitis without treatment ($p<0.05$). Production of IL-1 was not modulated by endodontic treatment ($p>0.05$). Periapical healing was observed in approximately 60% of the cases after endodontic treatment performed in a single session with lower synthesis of TNF- α and OPN in the periapical region, regardless of the irrigation protocol used.

Key Words: apical periodontitis, single visit root canal treatment, apical negative pressure irrigation, positive pressure irrigation, passive ultrasonic irrigation.

Introduction

Apical periodontitis represents a localized immune-inflammatory response against the microorganisms emerging from the root canal and is characterized by the presence of a mixed inflammatory infiltrate, composed of T and B lymphocytes, neutrophils, macrophages and plasma cells, depending on the stage of the disease (1-3). If pathogens are not eliminated, the disease will progress to destruction of the mineralized tissues around the root apex (4). In this scenario, several inflammatory mediators are produced locally to regulate the immune system, i.e. the recruitment of inflammatory cells and their activity (5-7).

The recruitment of inflammatory cells to the apical and periapical regions is directed by the release of chemotactic factors, such as the cytokines interleukin-1 α (IL-1 α) and tumor necrosis factor- α (TNF- α) (6,7), which locally induce inflammation and bone tissue resorption (6-9). Osteopontin (OPN) is a phosphoprotein secreted by T lymphocytes and macrophages, important for both the regulation of the immune response - recruitment of leukocytes and

activation of dendritic cells - and mediation of bone resorption (10-12).

Clinically, biomechanical preparation of the root canal associated with irrigation with an antimicrobial solution is a fundamental step of the endodontic treatment with the aim of reducing the levels of microorganisms and their by-products in the root canal system (13). However, regardless of instrumentation and irrigation techniques used, some areas remain inaccessible, maintaining microbial contamination (13,14).

Conventional irrigation by positive pressure is the most common method in endodontic treatment, but with this technique there is a greater possibility of extrusion of the irrigating solution to the periapical region, which may result in periapical tissue injury, postoperative pain and delay in the repair process (15). In order to overcome these limitations, passive ultrasonic irrigation was developed to improve antisepsis of the root canal system during instrumentation (16,17). In primary endodontic infections, passive ultrasonic irrigation showed efficacy

in removing bacteria from the root canals and was more efficient than the positive pressure (17). Another irrigation protocol of the root canal system occurs through negative apical pressure, which allows the use of a larger volume of irrigation solution and present a greater potential of cleaning and elimination of the microbial content of the root canals with less extrusion of irrigating solution for the periapical tissues (18). Recently, it has been demonstrated that this system allows a high depth of penetration of the irrigating solution in the dentinal tubules, although this effect was higher in the cervical region compared to the middle and apical thirds (19).

Previously, it was demonstrated *in vivo* that irrigation by negative apical pressure, passive ultrasonic and positive pressure does not remove all microbial content present in the root canals, but the negative apical pressure allows a mild inflammatory response in the periapical region, after treatment in a single session in teeth with apical periodontitis (20). However, the mechanism involved in inflammatory infiltrate reduction observed after apical negative pressure irrigation or in the maintenance of inflammation on positive pressure or passive ultrasonic irrigation is not clear. Therefore, the present study was carried out to evaluate (i) the periapical repair in teeth with apical periodontitis after the use of conventional irrigation by positive pressure, irrigation by negative apical pressure and passive ultrasonic irrigation by means of conventional periapical radiography; and (ii) the expression of inflammatory mediators in the periapical region by means of immunohistochemical evaluation. The null hypothesis of this study was that no difference would be found in apical periodontitis healing following root canal treatment regardless of the irrigation system used.

Material and Methods

Acquisition of Slides and Periapical Radiographs

The slides and periapical radiographs from 40 teeth (80 root canals) used in this study were obtained from the specimen database at the Department of Pediatric Dentistry at the School of Dentistry of Ribeirão Preto (University of São Paulo, Brazil). Detailed methodology was reported previously by Cohenca et al. (20). Experiments were performed in accordance with ISO 7405:2008 and the study was approved by the Institutional Animal Research Ethics Committee (process #014/2012).

After coronal pulp exposure, the pulp tissue was extirpated, and the apical cementum layer was perforated with the sequential use of size #15 up to #30 K-files, thus creating standardized apical openings. The root canals were left exposed to the oral cavity for 7 days. After this period, the pulp chamber was sealed for 45 days with zinc oxide and eugenol cement (SS White, Rio de Janeiro, RJ,

Brazil) to induce apical periodontitis as previously described (21,22). Prior to performing root canal treatment, apical periodontitis development was confirmed radiographically. Then, the teeth were isolated using a rubber dam, working length was established 1 mm short of the radiographic apex and confirmed by using an electronic apex locator (Root ZXII, J Morita Corp., Kyoto, Japan). The root canals were instrumented with ProTaper Universal NiTi rotary systems (Dentsply/Maillefer, Ballaigues, Switzerland) powered by the X-Smart™ endodontic micromotor (Dentsply/Maillefer). Irrigation of the canal system was performed with 2.0 mL of 5.25 % sodium hypochlorite (NaOCl) at each instrument change. During instrumentation, each root canal was irrigated according to the protocol established for each group as follows: Group 1 - apical negative pressure irrigation (EndoVac® system; Discus Dental, Culver City, CA, USA) throughout canal instrumentation. Final irrigation sequence was performed using 30 s of 5.25 % NaOCl (macroirrigation), 30 s of 17 % EDTA (microirrigation), and 30 s of 5.25 % NaOCl (microirrigation). Group 2 - irrigation via a 30-G standard needle (Max-i-Probe; Dentsply/Tulsa, Tulsa, OK, USA). Final irrigation sequence was performed using 30 s of 5.25 % NaOCl followed by passive ultrasonic irrigation for 20 s, 30 s of 17 % EDTA followed by passive ultrasonic irrigation for 20 s, and 30 s of 5.25 % NaOCl followed by passive ultrasonic irrigation for 20 s. Passive ultrasonic irrigation was performed using Irrisafe tips (Satelec, Acteon Group Merignac Cedex, France), mounted on a Neutron XS P5 ultrasonic unit (Satelec), set at 10 power. Group 3 - irrigation using a 30-G standard needle and conventional irrigation using positive pressure. The final irrigation sequence was performed using 30 s of 5.25% NaOCl, 30 s of 17% EDTA, and 30 s of 5.25% NaOCl.

All root canals were filled with gutta-percha and AH-Plus cement (Dentsply De Trey, Konstanz, Germany), canal orifices were sealed with MTA ProRoot (Dentsply Tulsa Dental, Johnson City, TN, USA), and the teeth were restored with silver amalgam (Sybraloy; Kerr Corporation, Orange, CA, USA). On Group 4, apical periodontitis was induced but root canal treatment was not performed.

Radiographic Evaluation

The periapical status was characterized according to the Periapical Index (PAI), using standardized periapical radiographs performed prior to and 180 days following root canal treatment. For that purpose, the radiographs were distributed at random to two calibrated and blind examiners (Kappa = 0.82) who assigned a score according to PAI: (1) normal periapical structure, (2) minor changes in bone structure, (3) change in bone structure with some mineral loss, (4) periodontitis with a well defined radiolucent area, (5) severe periodontitis with exacerbated features.

The scores obtained for each group prior to and following root canal treatment were compared using Wilcoxon signed-rank test ($\alpha = 5\%$). A dichotomized analysis was also performed where PAI 1 and 2 were considered success whereas PAI 3, 4 and 5 were considered failure. Intergroup comparison was performed using Fisher Exact test ($\alpha = 5\%$).

Immunohistochemistry

The slides were deparaffinized, hydrated in a decreasing ethanol series, and kept in phosphate-buffered saline (PBS). Next, tissue sections were microwaved (7 x 12 seconds at 2-min intervals) with sodium citrate buffer (pH = 6.0) for antigen retrieval. After temperature stabilization, the slides were washed with PBS (3x) for 5 min, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. Slides were further washed with PBS (3x) for 5 min, and nonspecific binding sites were blocked with 5% bovine serum albumin (Sigma-Aldrich) for 30 min. The tissues were then incubated with primary antibodies for TNF- α (1:200; ab6671 - rabbit polyclonal; Abcam Inc., Cambridge, MA, USA), IL-1 α (1:100; PAA071Ca01 - rabbit polyclonal; Cloud-Clone Corp., Katy, TX, USA), and OPN (1:200; ab8448 - rabbit polyclonal; Abcam Inc.) at 4 $^{\circ}$ C overnight. Next, slides were washed and incubated with biotinylated secondary antibodies (1:1,000) for 1 hour, washed in PBS, and incubated with streptavidin conjugated with horseradish peroxidase for 30 min; 3,3-diaminobenzidine (Sigma-Aldrich) was used as the enzyme substrate for 5 minutes. The slides were washed with PBS, counterstained with Harris's hematoxylin for 15 s, washed with distilled water, dehydrated in increasing ethanol concentrations, and mounted in Entellan (Merck, Darmstadt, Germany). Control slides in which the primary antibody was omitted were used to test the specificity of immunostaining. Positive staining in periapical tissues was scored (1= mild, 2= moderate, and 3= intense) as previously described (23). For this analysis, all slides were prepared in the same batch to obtain a standardized staining and were evaluated by an experienced blind examiner. Data were converted into percentage and intergroup comparison was performed using Kruskal-Wallis followed by Dunn's post test ($\alpha = 5\%$).

Results

Radiographic Evaluation

After contamination of the root canals, the specimens of all groups showed discontinuity of the lamina dura and presence of radiolucent areas suggestive of apical periodontitis. At 180 days after the endodontic treatment, the radiographic examination showed the persistence of periapical radiolucent areas and discontinuity of the lamina dura in 35% of the specimens of negative apical pressure

group, 40% of the specimens of the passive ultrasonic group and 40% of the specimens of the positive pressure group. There was no difference between the groups, regardless of the irrigation protocol used ($p > 0.05$). When compared to radiographs prior to treatment, in all groups, a reduction in the size of apical periodontitis and the presence of denser bone trabeculate ($p < 0.05$) could be observed, but there was no difference among the irrigation protocols used ($p > 0.05$) (Fig. 1).

Immunohistochemical Evaluation

Regarding the evaluation of OPN (Figs. 2A and 3) and TNF- α (Figs. 2B and 4), a more intense staining was observed in teeth with apical periodontitis without treatment than in the teeth submitted to the different irrigation protocols ($p < 0.05$). However, there was no statistically significant difference among the different irrigation protocols ($p > 0.05$). The staining for IL-1 α (Figs. 2C and 5) did not differ between the group with apical periodontitis without treatment compared to the groups submitted to different irrigation protocols ($p > 0.05$).

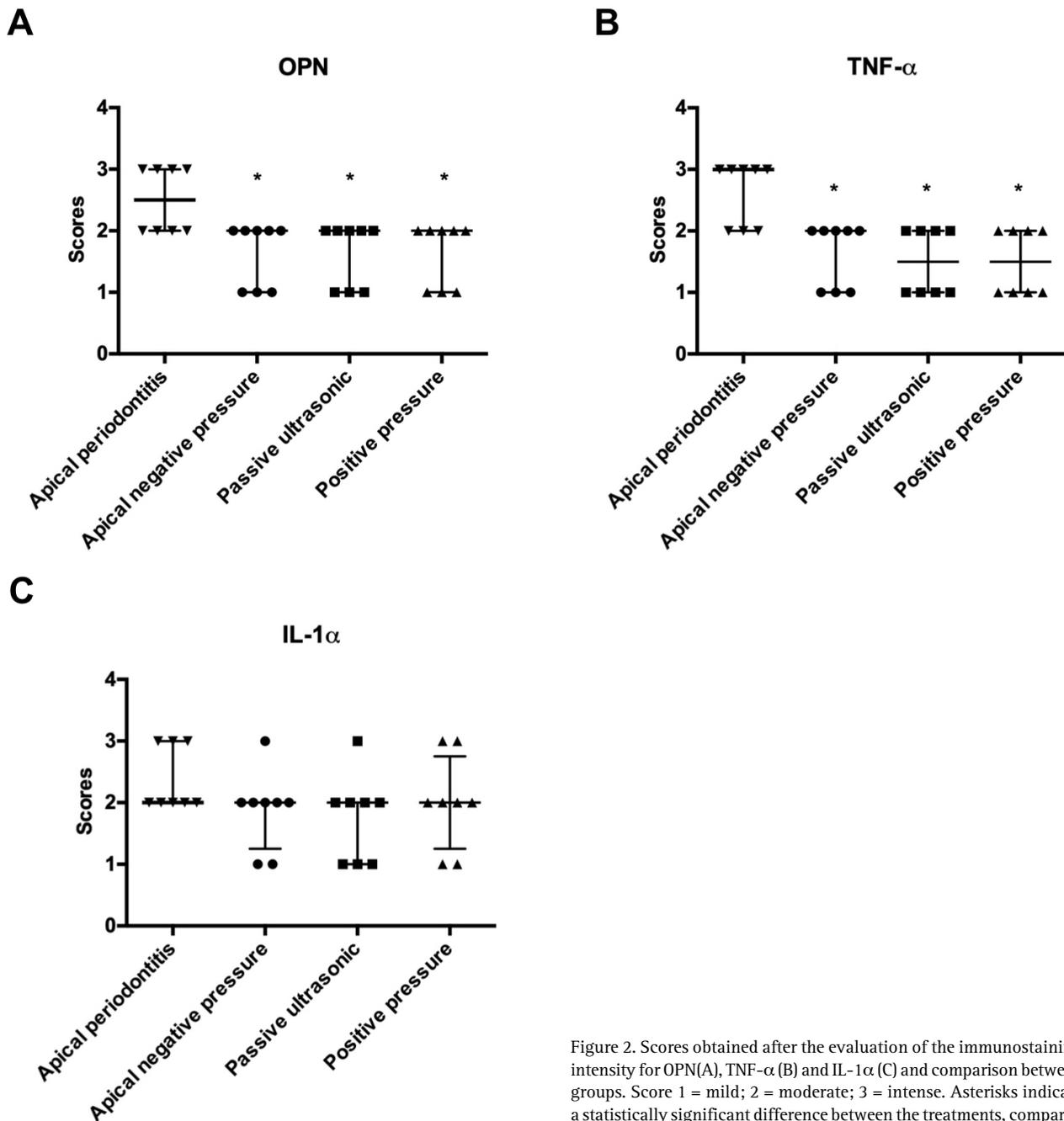
Discussion

Several irrigation techniques and devices have been used to improve antisepsis of the root canal system. In the present study radiographic evaluation of the repair in teeth with apical periodontitis after endodontic treatment using 3 different irrigation systems as well as the response of the apical and periapical tissues to identify inflammatory mediators in the region was investigated. In general, it was observed that after 180 days of endodontic treatment, performed in a single session, there was radiographic repair of the apical periodontitis in about 60% of the cases, regardless of the irrigation system used. Radiographic examination in several studies has been used to determine the success of endodontic treatment in teeth with or without apical periodontitis (22). In this study, PAI was used as a tool to detect periapical *status*, based on previous clinical and epidemiological studies that aimed to evaluate changes in the extent and severity of the apical periodontitis after endodontic treatment (24,25). The increasing of the size of periapical radiolucency on radiograph after endodontic treatment indicates failure, whereas reduction or absence means repair. As used in this study, PAI can be used dichotomously in success (PAI 1 and 2) and failure (PAI 3, 4 and 5) (26). However, radiographic analysis should be considered limited and does not accurately depict three-dimensional morphological changes of apical lesions (22,27), and may underestimate or overestimate the extent of apical periodontitis with the same volume resulting in divergent classifications of periapical status (25).

For this reason and considering the absence of repair in

40% of the cases, the microscopic evaluation of the tissues was necessary. Although the radiographic evolution of the apical periodontitis is relatively well known, the molecular nature of the persistent lesions is little explored. Regarding the inflammatory response, a lower production of the inflammatory mediators TNF- α and OPN was identified in the periapical region after the use of the different irrigation protocols compared to the apical periodontitis without treatment, but with no difference between the irrigation protocols used during endodontic treatment.

In teeth with incomplete root formation, after irrigation of root canals with negative apical pressure, there was repair of the lesion with reduction of inflammation and new bone formation (28,29). In teeth with complete root formation, a milder inflammatory response was observed (20), corroborating the reduction in the production of inflammatory mediators observed in this study, although bone repair was delayed. Absence of apical and periapical repair were also reported after biomechanical preparation using positive pressure irrigation and root canal filling in



Root canal treatment and irrigation systems

Figure 2. Scores obtained after the evaluation of the immunostaining intensity for OPN(A), TNF- α (B) and IL-1 α (C) and comparison between groups. Score 1 = mild; 2 = moderate; 3 = intense. Asterisks indicate a statistically significant difference between the treatments, compared to apical periodontitis without treatment.

teeth with apical periodontitis in a single session (13,22).

In previous studies, it was found that root canal treatment in a single visit, in teeth with apical periodontitis, do not eliminate bacterial load in the root canal system (30). Histologically, single visit root canal treatment using 2.5% sodium hypochlorite irrigation allowed the persistence of bacteria resulting in a chronic inflammatory infiltrate

surrounding the tooth apex with active bone resorption (31,32). Also, even using 5.25% sodium hypochlorite irrigation, bacterial load was not eliminated nor bone resorption impaired (20,33). Here, it was demonstrated that apical periodontitis persistence after single-visit treatment is sustained by a pro-inflammatory milieu with the presence of inflammatory cells and mediators.

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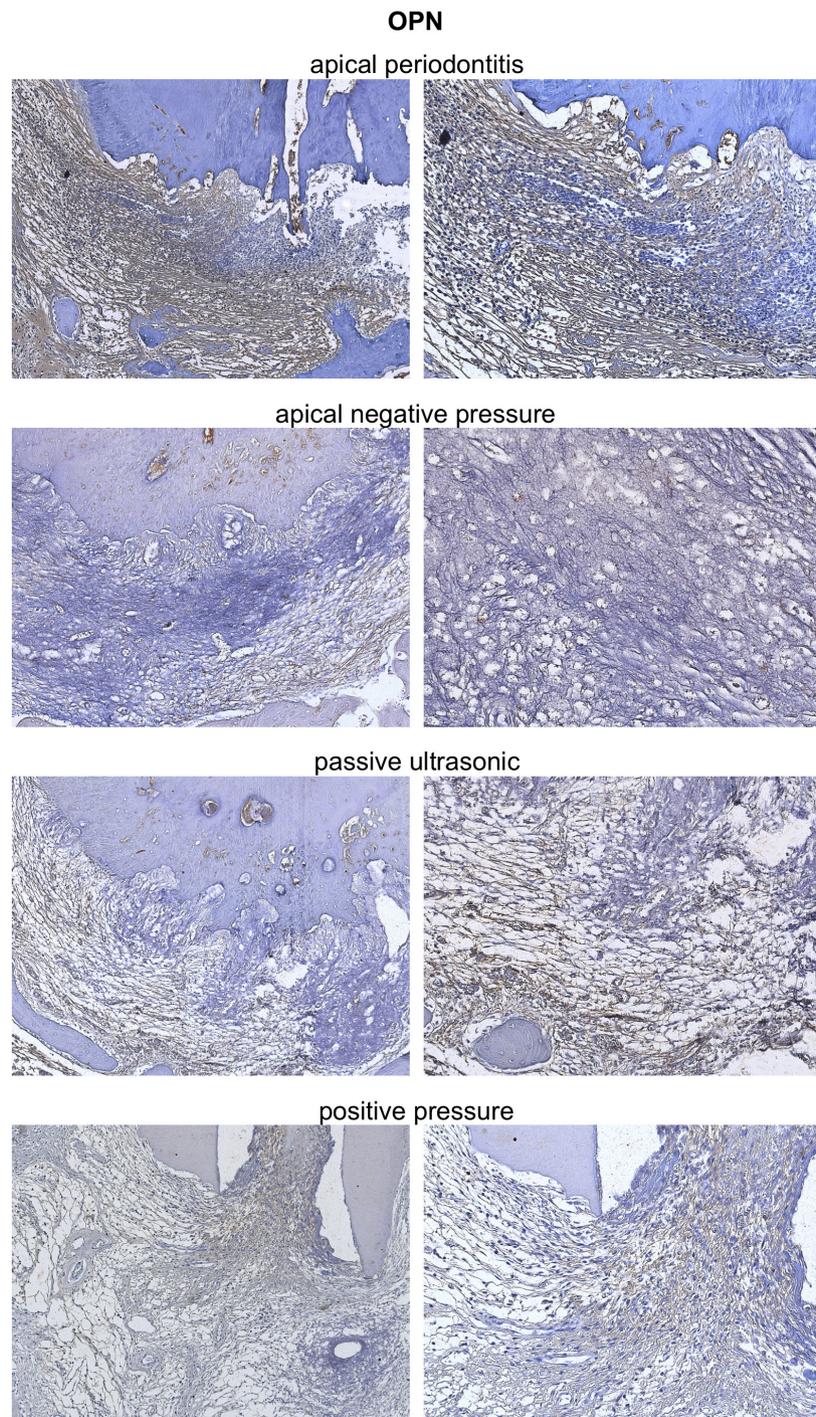


Figure 3. Representative photomicrographs of the immunostaining for OPN in the periapical region of teeth with apical periodontitis without treatment and after endodontic treatment using different irrigation protocols. Original magnification of 10x (left) and 20x (right).

The use of passive ultrasonic irrigation results in a better debridement condition of the root canal system, greater effectiveness in the irrigation solution release in working length and greater smear layer removal compared to positive pressure irrigation (16). *In vivo*, in a randomized clinical study in humans, passive ultrasonic irrigation was effective in reducing the number of bacteria, but

was not able to remove bacterial endotoxin (17), which may represent an important factor for the persistence of apical periodontitis refractory to endodontic treatment, as observed in the present study. Taken together, these results indicate that the effectiveness of irrigation methods in the removal of bacterial products and by-products must be further explored in the future.

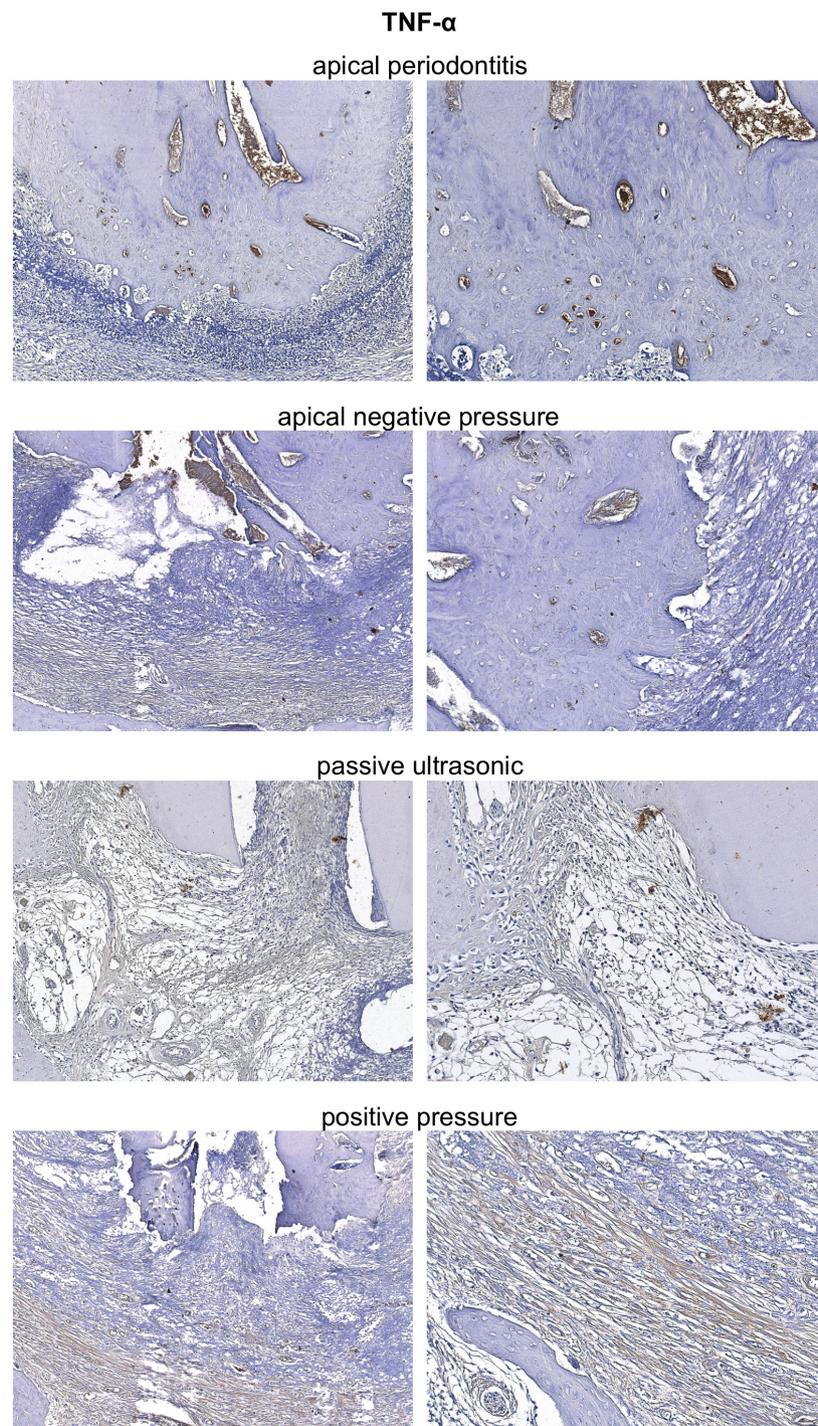


Figure 4. Representative photomicrographs of the immunostaining for TNF- α in the periapical region of teeth with apical periodontitis without treatment and after endodontic treatment using different irrigation protocols. Original magnification of 10 \times (left) and 20 \times (right).

OPN and TNF- α production were more intense in the teeth with apical periodontitis without treatment, but it was not possible to find a statistically significant difference between the groups submitted to the different irrigation protocols. In bone tissue, the role of osteopontin is controversial, since the molecule can function as an osteoclast anchoring protein by increasing bone catabolism (10,12,34) or as a protective factor in the recruitment of

phagocytes to polymicrobial infection (35). Significantly higher levels of TNF- α have also been observed in more active lesions, characterized by increased expression of the activator receptor ligand of the nuclear factor kappa B (8).

On the other hand, IL-1 α was not modulated by endodontic treatment. Since this proinflammatory cytokine is a key regulator of host responses to microbial infection, increasing resorption and inhibiting bone formation (6), the

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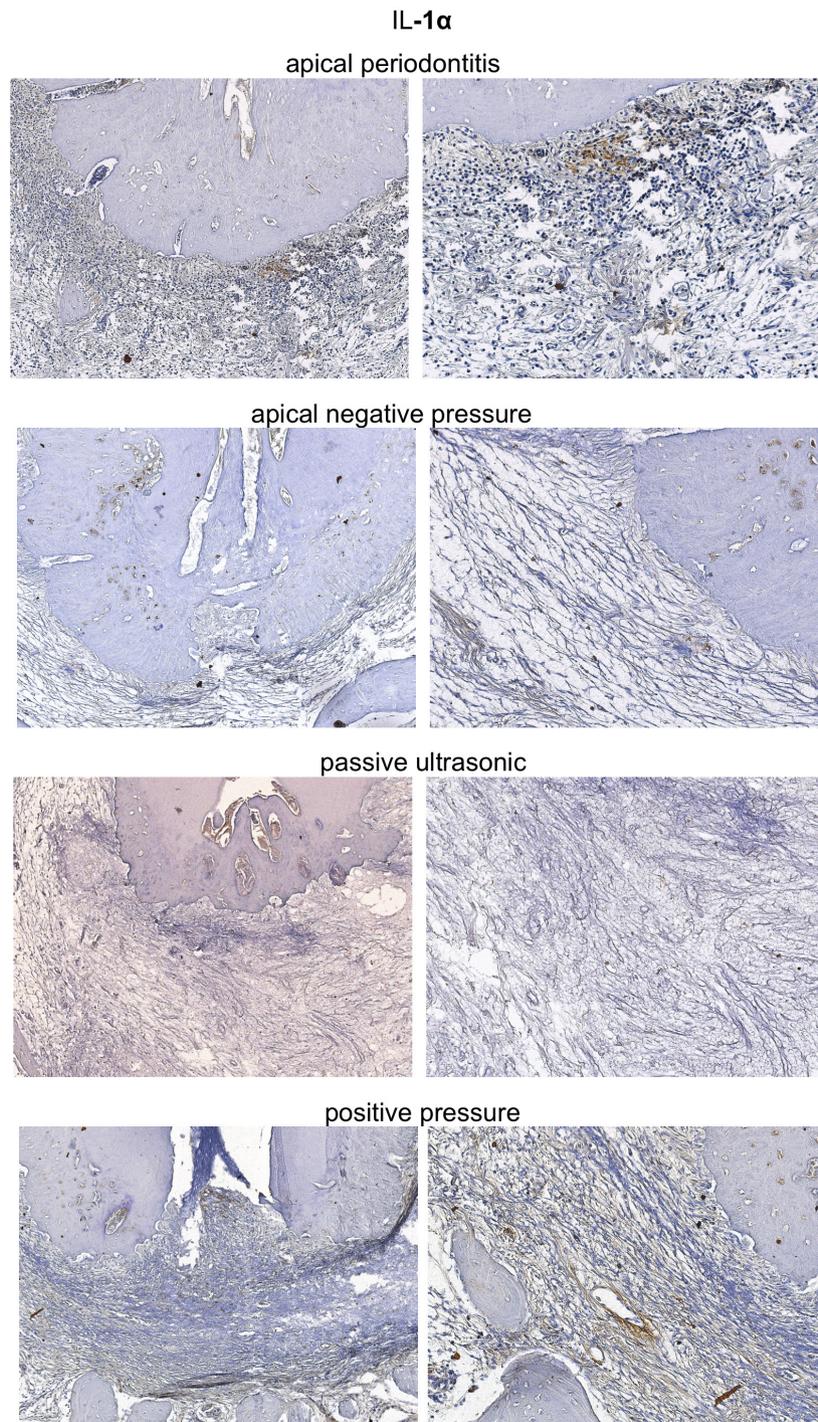


Figure 5. Representative photomicrographs of the immunostaining for interleukin-1 α in the periapical region of teeth with apical periodontitis without treatment and after endodontic treatment using different irrigation protocols. Original magnification of 10 \times (left) and 20 \times (right).

persistence of the lesion in this study may be correlated with the non-modulation of this cytokine by endodontic treatment. In apical periodontitis of deciduous human teeth, interleukin-1 α and interleukin-1 β were expressed by different cell types in granulomas and root cysts, such as inflammatory cells, epithelial cells and vascular endothelium (36).

In general, the results of this research indicate that there was partial apical periodontitis repair after endodontic treatment in a single visit, indicating that this protocol should not be used in teeth with apical periodontitis, regardless of the irrigation system used. Inadequate root canal cleaning may have been responsible for maintaining microorganisms and their by-products in the root canal system, stimulating the host's immune response. In this periapical immune-inflammatory response, inflammatory mediators that positively regulate bone resorption were produced, which resulted in a persistent lesion 180 days after root canal contamination. Unfavorable outcomes after endodontic treatment in this study can be related to the absence of an antimicrobial intracanal dressing between sessions. However, in this study treatment was performed in a single session, aiming to investigate the comparative efficacy analysis of different irrigation systems. Further studies should be conducted to investigate whether these irrigation protocols in combination with root canal dressing would raise the success rate of endodontic treatment in teeth with apical periodontitis.

Apical periodontitis repair was observed in approximately 60% of the cases after endodontic treatment performed in a single session with a lower synthesis of TNF- α and OPN in the periapical region, regardless of the irrigation protocol used (negative apical pressure, passive ultrasonic and by positive pressure). The production of interleukin-1 α was not modulated by endodontic treatment.

Resumo

O objetivo deste estudo foi avaliar o reparo periapical e a síntese de mediadores inflamatórios após tratamento endodôntico em dentes de cães com lesão periapical, em sessão única, utilizando diferentes protocolos de irrigação. Lesões periapicais foram induzidas experimentalmente em dentes de cães e aleatoriamente divididas em 4 grupos: G1 - Irrigação por Pressão Apical Negativa (n = 20); G2 - Irrigação Ultrassônica Passiva (n = 20), G3 - Irrigação por Pressão Positiva (n = 20); G4 - Lesão periapical sem tratamento (n = 20). Após 180 dias, os animais foram eutanasiados, as peças removidas e submetidas ao processamento histotécnico para análise imunohistoquímica para osteopontina (OPN), fator de necrose tumoral- α (TNF- α) e interleucina 1- α (IL-1 α). A análise radiográfica do reparo das lesões periapicais foi realizada por meio do Índice Periapical, obtido antes e 180 dias após o tratamento endodôntico. Os resultados obtidos foram submetidos à análise estatística por meio dos testes de sinais de Wilcoxon, Exato de Fisher ou Kruskal-Wallis seguido pelo pós-teste de Dunn (α = 5%). O exame radiográfico após o tratamento endodôntico, mostrou a persistência de áreas radiolúcidas periapicais e descontinuidade da lâmina dura em 35% dos espécimes do G1, 40% do G2 e 40% do G3, embora uma redução no PAI tenha sido observada ($p < 0,05$). Pela análise imuno-

hitoquímica, o tratamento endodôntico resultou na menor síntese de TNF- α e de OPN na região periapical, comparativamente à lesão periapical sem tratamento ($p < 0,05$). A produção de IL-1 α não foi modulada pelo tratamento endodôntico ($p > 0,05$). Reparo da lesão periapical foi observado em cerca de 60% dos casos após tratamento endodôntico realizado em sessão única e menor síntese de TNF- α e de OPN na região periapical, independente do protocolo de irrigação utilizado.

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