Comparison of apical periodontitis repair in endodontic treatment with calcium hydroxide-dressing and aPDT

Abstract: This study evaluated the effect of antimicrobial photodynamic therapy (aPDT) on the endodontic treatment of apical periodontitis (AP). AP was induced in 48 premolars of 6 dogs. After biomechanical preparation, the teeth were divided into 4 groups: Calcium-Hydroxide (CH)/120d and CH/180d: root canals filled with CH-based dressing for 15 days before obturation; aPDT/120d and aPDT/180d: conditioning with phenothiazine photosensitizer (10 mg/mL) for 1 minute and irradiation with diode laser in the same session as obturation. Root filling was performed with AH Plus sealer. After the experimental periods, animals were euthanized and teeth were submitted for histology. HE staining was performed for descriptive analysis of the periapical region, measurement of apical periodontitis and for inflammatory cells, and blood vessels count. Immunohistochemistry was performed for osteopontin (OPN) and alkaline phosphatase (ALP). Data were analyzed statistically by two-way ANOVA and chi-square test ($\alpha = 5\%$). Teeth in Group CH/120d presented only a slightly enlarged periodontal ligament (PL) with advanced repair. Group aPDT/120d presented the PL moderately enlarged, with moderate inflammatory infiltrate and few collagen fibers. The same pattern was observed at 180 days. AP lesions in CH-treated groups were smaller than those in aPDT-treated groups ($p < 0.001$) with more blood vessels ($p < 0.0001$), regardless of the evaluation period, without significant differences in the number of inflammatory cells ($p > 0.05$). CH-treated groups showed significantly more intense immunostaining for ALP and OPN ($p < 0.001$) in both periods. Although aPDT stimulated angiogenesis and expression of bone formation markers, the two-session endodontic treatment with CH-based dressing promoted better apical periodontitis repair.

Keywords: Photochemotherapy; Periapical Periodontitis; Calcium Hydroxide; Endodontics.

Introduction

The success of endodontic treatment depends on many factors and can be hampered by the complex anatomy of root canals including their number, curvature and ramifications, and the location of the apical foramen. These factors hinder complete cleaning of the root canal system. Additionally, the presence of apical periodontitis as a consequence of infection is associated...
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with a higher failure rate after endodontic treatment. Therefore, an intracanal medication should be used in multiple sessions to improve disinfection of the root canal system during endodontic treatment of teeth with apical periodontitis. The success rates in these cases are higher with the use of an antibacterial intracanal medication in multiple sessions, compared to those with a one-session treatment.3,4,5

Calcium hydroxide has many biological properties, such as antimicrobial action and induction of mineralization and periapical repair, making it a highly recommended intracanal medication during endodontic treatment.6 There is clinical evidence showing that intracanal dressing with calcium hydroxide is indicated in cases of teeth with apical periodontitis, prior to the final root canal filling.7,8

Traditionally, endodontic treatment in multiple sessions with intracanal medication aims to reduce or eliminate microorganisms and their by-products from the root canal system prior to obturation. It is well accepted as a safe and common therapy.9 However, in recent years, besides some advantages like good patient acceptance and practical management considerations in the dental clinic offered by one session treatment,9 there is a growing divergence regarding the need for multiple sessions during endodontic therapy. Although several studies have reported non-significant differences between treatments performed in one and multiple sessions,10,11 it should be emphasized these reports are clinical and radiographic studies.5,10 These results should thus be considered with caution.

Currently, the search for complementary alternatives to one-session endodontic treatment is aimed to achieve a success rate comparable or better than with the use of intracanal medication in multiple sessions. Antimicrobial photodynamic therapy (aPDT) is an alternative that has been introduced in Endodontics and has been considered a promising treatment. Several in vivo and in vitro studies have shown that this therapy can significantly improve the endodontic treatment outcome specially by reducing microbial counts.4,12,13,14

aPDT involves the application of a photosensitizer, followed by a light source in the sensitized tissues, which generates a toxic reaction in the target cells, causing the death of microorganisms.15,16 Currently, aPDT is considered a complementary therapy to the conventional protocols used for disinfecting the root canal system.17 Some studies have evaluated the efficacy of aPDT in the endodontic treatment of teeth with apical periodontitis.5,12,14,18 However, there is still insufficient scientific evidence to safely indicate the use of aPDT in place of conventional treatment.5 Moreover, there are no studies in specific literature that evaluate the bone formation mediators and angiogenesis after endodontic treatment supplemented with aPDT.

Thus, this study aimed to evaluate the in vivo effect of one-session endodontic treatment with aPDT in the repair of apical periodontitis, compared to treatment in two sessions using a calcium hydroxide intracanal dressing in dogs.

**Methodology**

This study evaluated 48 teeth from the second and third maxillary premolars and the second, third and fourth mandibular premolars of six 12-month-old mongrel dogs, distributed into four groups. All animal methods were performed according to the Animal Research Ethics Committee of the School of Dentistry of Bauru, University of São Paulo - Brazil (Process number 19/2012).

**Operative procedures**

The protocol applied for the induction of apical periodontitis was described previously by Silva et al.12 The animals were anesthetized 15 minutes before the operative procedures and received inhalation anesthesia with isoflurane (Abbott Laboratories, St Laurent, QC, Canada). Then, the pulp tissues were removed and canals were exposed to the oral cavity for 7 days, when the coronal accesses were sealed with zinc oxide-eugenol cement to induce apical periodontitis. Radiographs were taken every 15 days until periapical radiolucencies were developed, which commonly occurred after 45 days.

Once apical periodontitis was established, the teeth were isolated with a rubber dam, the operative field was decontaminated with 2% chlorhexidine gluconate, and the necrotic content of the canals
was removed in a pressureless crown-down manner associated with 2.5% NaOCl in abundant irrigation. Size 15 to 25 K files (Dentsply Maillefer, Ballaigues, Switzerland) were used sequentially at the radiographic apex under irrigation at each instrument change in order to standardize the apical opening. A Working Length (WL) at 1 mm short of the radiographic apex was established. The canals were then instrumented using the ProTaper NiTi rotary Universal System powered by the X-Smart endodontic micromotor (Dentsply Maillefer) and were irrigated with 3.6 mL 2.5% NaOCl at each file change. After all instrumentation procedures, the canals were dried with sterile paper points and every hemi-arch of each animal was randomly subjected to different treatment protocols in order to test all variables in the same animal.

Then, four groups were established according to the following treatments:

- **Calcium-Hydroxide (CH)/120d and CH/180d** (two-session endodontic treatment with a CH-based dressing): After the instrumentation procedures, the root canals were filled with a commercial CH-based paste (Calen®; S.S. White Artigos Dentarios Ltda., Rio de Janeiro, Brazil) at the radiographic apex using a 27-G long needle. Teeth had the crown restored with a glass-ionomer-based cement for 15 days. After this period, the canal dressing was removed under rubber dam isolation by successive irrigation with 2.5% NaOCl. EDTA solution was applied at the end for 3 minutes. Root filling was performed using AH Plus sealer (De Trey; Dentsply, Konstanz, Germany) and gutta-percha cones by lateral condensation. The teeth were definitely restored with a glass-ionomer cement base and silver amalgam.

- **Groups aPDT/120d and aPDT/180d** (one-session endodontic treatment supplemented with aPDT): After the instrumentation procedure, the PS (phenothiazine chloride at 10 mg/mL concentration; 70 μL volume; HELBO Blue Photosensitizer; Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria) was applied inside the root canal with an endodontic needle and allowed to react for 1 minute. The root canals were then rinsed with sterile distilled water, dried with sterile paper points, and irradiated with a laser source. A 660-nm wavelength handheld battery-operated diode laser with 20 mW power output and 60 mW/cm² irradiance (HELBO Therapielaser, Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria) was applied. The laser was delivered through a flexible fiber-optic tip with 0.6 mm diameter (HELBO 3D Pocket Probe, Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria), with a fiberspot area equivalent to 0.002 cm², 0.06 W output power, and 3.6 J of energy. During the irradiation time, the fiber was left within the canal at the WL. The fiber was designed to provide a 3-D exposure of the interest area. The laser application covered 2 mm per second, applying a vertical movement from the apex toward the cervical region. The photosensitizer was irradiated during 1 minute in a continuous wave mode as recommended by the manufacturer. The fluence delivered to the PS was 1.800 J/cm². The canals were then irrigated with saline and dried with sterile absorbent paper points. Root filling was performed in the same session with AH Plus sealer and gutta-percha cones. The teeth were definitely restored as described for the other groups.

The animals were euthanized after 120 days (groups CH/120d and aPDT/120d) and after 180 days (groups CH/180d and aPDT/180d). The jaws with teeth were dissected and sectioned to obtain individual roots, which were fixed in 10% buffered formalin for 48 h, demineralized in EDTA, and embedded in paraffin. Sagittal serial sections with 5-μm thickness were obtained and stained with hematoxylin and eosin (HE). The sections were examined under light microscopy by an experienced and blinded examiner to the groups. A descriptive microscopic analysis was performed using the AXIO IMAGER.M1 microscope (Carl Zeiss, Jena, Germany) coupled to an AXIOCAM MRc5 camera (CarlZeiss, Jena, Germany).

**Descriptive analysis of apical and periapical region under conventional light microscopy**

The apical and periapical aspects of each group were described according to the following histopathological parameters: periapical inflammatory infiltrate, apical periodontal ligament thickness, and mineralized tissue resorption.5
Inflammatory cell count

The HE-stained sections were used to count the number of inflammatory cells (polymorphonuclear and mononuclear leukocytes) under conventional light microscopy, using their identifying characteristics. The analysis was performed using an Axio Imager.M1 microscope at 40X in a counting frame of approximately 0.085 mm², centered from the apical foramina in the medial region of the periapical lesion. Results were expressed as number of inflammatory cells.

Morphometric analysis of periapical lesion size under conventional light microscopy

Morphometric analysis of periapical lesion size was performed on the HE-stained specimens using an Axio Imager.M1 microscope at ×1.25 magnification. The periapical lesion area in mm², was delimited and measured using the Axio Vision Rel 4.8 software. Delimitation of the lesion excluded the healthy areas such as periodontal ligament, cementum, and alveolar bone, and included resorption areas and inflammatory infiltrates.

Angiogenesis analysis - Blood vessel count

The number of blood vessels in the medial region of the lesion adjacent to the apical foramen were measured in specimens stained with HE, in an area of approximately 0.085 mm². The analysis was performed at 40X magnification under conventional and fluorescent light, using the Alexa Fluor 488 filter (AF488), in the Axio Imager.M1 microscope.

Blood vessels were identified by the strong green fluorescence, evidencing both vessel walls and the red blood cells present therein. Results were expressed as the number of blood vessels.

Immunohistochemical analysis

Immunohistochemical analysis was used to determine the intensity of osteopontin (OPN) and alkaline phosphatase (ALP) immunostaining using the immunoperoxidase technique.

Histological sections were deparaffinized and the antigenic epitopes were recovered with heat using citrate buffer in a microwave oven. After returning to room temperature, the slides were washed 2 times for 5 minutes with PBS and 1 time with PBS/Triton solution (Sigma-Aldrich Corporation, St. Louis, USA) for 5 minutes. Endogenous peroxidase blockade was performed using 3% hydrogen peroxide for 40 minutes. Then the slides were washed again with PBS and PBS/Triton as described. Nonspecific binding blockade was performed with 3% BSA (bovine serum albumin)/PBS for 1 hour. Next, the slides were incubated overnight under refrigeration with primary antibodies diluted in 3% BSA: anti-OPN (rabbit polyclonal antibody, ab8448, Abcam PLC, diluted 1:150) and anti-ALP (mouse monoclonal antibody, sc-271431, Santa Cruz Biotechnology Inc., Santa Cruz, USA; diluted 1:50).

After returning to room temperature, the slides were washed and incubated with the biotinylated secondary antibody (goat anti-mouse IgG-B sc-2039 and goat anti-rabbit IgG-B sc-2040; Santa Cruz Biotechnology Inc., Santa Cruz, USA; diluted 1:200) for 1 hour. After washing, the avidin-biotin peroxidase complex (ABC kit, Vector Laboratories Inc., Burlingame, USA) was added for 30 minutes. The slides were washed again with PBS and PBS/Triton and the reaction was developed with 3,3’-diaminobenzidine solution (DAB; Sigma-Aldrich Corporation, St Louis, USA) and 3% H₂O₂ in PBS for 1 minute. The slides were counterstained with Harris hematoxylin for 10 seconds, washed in running water, washed in ammonium water for 30 seconds, washed in running water, cleared, dehydrated, and mounted.

Analysis was performed with the Axio Imager.M1 microscope under conventional light. The following scores were attributed to positive immunostaining for OPN or ALP in the apical and periapical region: absent (0), mild (1), moderate (2), or intense (3).

Statistical analysis

Data from inflammatory cell count morphometric analyses and evaluation of blood vessels were analyzed statistically by two-way ANOVA. The immunohistochemical data were transformed into percentage and analyzed by the chi-square test. Graph Pad Prism 4.0 statistical software (Graph Pad Software Inc., San Diego, USA) was used for all tests and a significance level of 5% was adopted.
Figure 1. Histopathological events observed after the evaluation of periapical tissue response to the proposed endodontic treatments. (A) Tooth in Group treated with calcium hydroxide (CH) after 120 days: periodontal ligament slightly enlarged with an advanced repair process, abundant collagen fiber and inflammatory cells scarce and diffuse. (B) A in highest magnification. (C) Tooth in Group treated with aPDT after 120 days: nonrepaired resorption areas, periodontal ligament more enlarged, intense inflammatory infiltrate, and few collagen fiber. (D) C in highest magnification. (E) Group CH/180d with the same characteristics as the group CH/120d. (F) E in highest magnification. (G) Group aPDT/180d with the same characteristics as the group aPDT/120d. (H) G in highest magnification.
Results

Descriptive analysis of apical and periapical regions

At 120 days, the apical cement of teeth in Group CH/120d presented the resorption areas repaired in most specimens. The periodontal ligament was only slightly enlarged with an advanced repair process and abundant collagen fibers. The presence of inflammatory cells was scarce and diffuse. In this same period, the specimens in Group aPDT/120d presented some nonrepaired resorption areas in the apical cementum. The periodontal ligament was moderately enlarged. The inflammatory infiltrate was mild and predominantly mononuclear, and few collagen fibers were observed. The alveolar bone was frequently denuded. The same pattern was observed at 180 days.

Figure 1 shows the histopathological events observed after evaluation of the periapical tissue response to the proposed endodontic treatments.

Inflammatory cell count

Statistical analysis of the number of inflammatory cells did not reveal a significant difference between the groups (p = 0.4), as shown in Table 1.

Morphometric analysis of periapical lesion size

The periapical lesions of groups treated with the CH-based paste were lower compared to the lesions treated with aPDT (p < 0.001), regardless of the treatment time. The time effect was not considered significant (p = 0.86). The periapical lesions of groups CH/120d, aPDT/120d, CH/180d, and aPDT/180d presented the areas: 1.18 (±0.41), 3.29 (±1,07), 1.41 (0.47), and 3.25 (2.97) mm², respectively (Figure 2).

Angiogenesis analysis - Blood vessel count

Statistical analysis after the evaluation of blood vessels (Figure 3) revealed statistically significant differences between groups (p < 0.0001). The specimens in groups treated with CH-based paste had more blood vessels compared to specimens in groups treated with aPDT, regardless of the evaluation period. Figure 4 shows the representative

Table 1. Statistical results of the number of inflammatory cells.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CH/120d</th>
<th>aPDT/120d</th>
<th>CH/180d</th>
<th>aPDT/180d</th>
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<tr>
<td>25% Percentile</td>
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<td>4.00</td>
<td>10.00</td>
</tr>
<tr>
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<td>31.50</td>
<td>6.00</td>
<td>58.50</td>
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<tr>
<td>Std. Deviation</td>
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<td>30.34</td>
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<tr>
<td>p-value</td>
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</table>

Figure 2. Periapical lesion area, in mm², after one-session endodontic treatment with aPDT or two-session endodontic treatment with a calcium hydroxide (CH)-based dressing after 120 or 180 days.

Figure 3. Number of blood vessels in the medial region of the periapical lesion adjacent to the apical foramen, after one-session endodontic treatment with aPDT or two-session endodontic treatment with a calcium hydroxide (CH)-based dressing after 120 or 180 days.
Figure 4. Representative photomicrographs of the specimens after two-session endodontic treatment with a calcium hydroxide (CH)-based dressing after 120 (AB) or 180 days (EF), and one-session endodontic treatment with aPDT after 120 (CD) and 180 days (GH), showing blood vessels present in the medial region of the periapical lesion adjacent to the apical foramen, in conventional and fluorescent light.
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specimens of each group in conventional and fluorescent light, evidencing the blood vessels present in the medial region of the periapical lesion adjacent to the apical foramen.

In addition, the blood vessels of specimens treated with CH-based paste were larger and mature, with thicker walls and a greater number of red blood cells inside, regardless of the evaluation period, as can be seen in Figure 4.

**Immunohistochemical analysis**

Statistical analysis after evaluation of immunostaining for ALP revealed that groups treated with CH-based paste showed significantly more intense immunostaining ($p < 0.001$) compared to aPDT, in both periods. Considering the intensity of positive immunostaining for ALP (Figure 5) as mild, moderate, and intense, we observed the following percentages, respectively: 0%, 40%, and 60% to group CH/120d; 20%, 80%, and 0% to aPDT/120d; 0%, 60%, and 40% to CH/180d; and 40%, 40% and 20% to aPDT/180d (Table 2).

The same response was observed after immunostaining for OPN (Figure 6), with more intense immunostaining in groups treated with CH-based paste ($p < 0.001$), in both periods. Considering the intensity of positive immunostaining for OPN as mild, moderate, and intense, we observed the following percentages, respectively: 20%, 60%, and 20% to group CH/120d; 60%, 40%, and 0% to aPDT/120d; 20%, 60%, and 20% to CH/180d and 40%, 60%, and 0% to aPDT/180d (Table 3).

Briefly, all treatments induced the expression of ALP and OPN, but more strongly in the CH-treated groups.

<table>
<thead>
<tr>
<th>Table 2. Data of the immunohistochemical analysis of ALP – percentage of each score in each group and p-value.</th>
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<td>Groups</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>CH/120d</td>
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<tr>
<td>aPDT/120d</td>
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<tr>
<td>CH/180d</td>
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<td>aPDT/180d</td>
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**Discussion**

This study evaluated the response of dog teeth with apical periodontitis to one-session endodontic treatment using aPDT or two-sessions after dressing with a CH-based medication.

The canine experimental model used in the present study simulates clinical situations such as oral microbiota, salivary flow, dental morphology, masticatory force, and host response. In addition, dogs are a long-validated study model (ISO 7405: 2008) specially because of their morphological and biological similarities with humans in the process of pulpal and periapical repair. Further, it is known that there is no direct correlation between ages of humans and dogs, as dogs have a faster development. Thus, the proposed time of 4 months for evaluating dog apical and periapical repair is equivalent to approximately 4 years in humans, being a suitable period for endodontic treatment evaluation. Thus, similar repair processes can be observed in shorter periods of time in dogs. In addition, this period was based on previous studies from our research group.

The groups of two-session treatment with CH-based medication showed the best results. The mechanism of action of calcium hydroxide that allows tissue repair is well known. Calcium hydroxide is chemically classified as a strong base with a high pH. The ionic dissociation of $\text{Ca}^{2+}$ and $\text{OH}^{-}$ ions is responsible for its antimicrobial properties and the induction of hard-tissue deposits. Some *in vivo* studies have shown the efficacy of calcium hydroxide against endodontic bacteria. Further, CH paste is well tolerated by bone and dental pulp tissues and has been the material of choice to create a calcified barrier in non-vital open-apex teeth. Therefore, in the present study, calcium hydroxide was considered as a control, since this protocol is known to provide higher success rates and to promote periapical repair in histopathological studies.

Histopathological results indicating tissue repair in groups treated with calcium hydroxide were confirmed by morphometric analysis of the periapical lesion size. Teeth in groups treated with CH-based paste presented significantly smaller lesions than
Figure 5. Representative photomicrographs of the specimens treated with two-session endodontic treatment with a calcium hydroxide (CH)-based dressing after 120 (A-B) and 180 days (E-F), and one-session endodontic treatment with aPDT after 120 (C-D) and 180 days (G-H), showing alkaline phosphatase [ALP] immunostaining (brown), which was more intense in CH-treated groups.
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Figure 6. Representative photomicrographs of the specimens treated with two-session endodontic treatment with a calcium hydroxide (CH)-based dressing after 120 (A-B) and 180 days (E-F), and one-session endodontic treatment with aPDT after 120 (C-D) and 180 days (G-H), showing osteopontin [OPN] immunostaining (brown), which was more intense in CH-treated groups.
those treated with aPDT after 120 and 180 days. These results are in agreement with previous studies that showed smaller lesions in groups medicated with a CH-based paste after 90, 120, and 180 days.

Although some in vitro studies have demonstrated a high antimicrobial effect of aPDT against endodontic pathogens and some in vivo studies have shown the efficacy of calcium hydroxide and aPDT against endodontic bacteria, previous histopathological evaluations are not consistent in recommending the use of aPDT to replace the conventional treatment in two sessions, which is in agreement with the present study. Therefore, the results indicate that the parameters for the safe use of aPDT under clinical conditions need to be better established.

Previous studies evaluating the histopathological periapical response after the use of aPDT at shorter periods (90 days or shorter) hypothesized that repair could be achieved after a longer evaluation periods. Therefore, Hidalgo et al. (2016) applied longer evaluation periods (120 and 180 days), showing that the treatment using a calcium hydroxide intracanal dressing in two sessions provided higher success rates than a single session with aPDT even after longer periods, in the same manner as in the present study.

Additionally, the present study focused on studying the mechanisms of action involved in periapical repair after endodontic treatment in one-session with aPDT or two-sessions with calcium hydroxide dressing. Therefore, it evaluated angiogenesis and the mediators of bone formation (ALP and OPN) post-treatment. Both treatments induced angiogenesis and the expression of these mediators, with greater intensity in the groups treated with calcium hydroxide paste.

ALP is a marker of osteoblastic activity, considered the most common indicator of bone formation. OPN is related to the late stage during osteoblastic differentiation, and exerts a protective effect in endodontic infections.

The effect of aPDT was previously evaluated in conjunction with periodontal treatment during periodontal wound healing. The mRNA levels for bone markers including ALP and OPN, were investigated and aPDT treatment was not able to increase the expression of these markers. There are no studies evaluating the expression of ALP and OPN in teeth with apical periodontitis after treatment with aPDT, which makes it difficult to compare our findings with previous studies.

Additionally, it is known that angiogenesis is essential for new tissue formation in the process of repair, allowing cells to receive the necessary nutrients for their proliferation and differentiation.

Therefore, blood vessel formation has been associated with more advanced tissue repair in experimental models in vivo. Borsatto et al., in a qualitative analysis of apical periodontitis treated with aPDT or calcium hydroxide dressing, demonstrated that calcium hydroxide induced greater proliferation of blood vessels, corroborating with the present study that confirmed this finding quantitatively.

According to a systematic review, limited clinical information is available on the use of aPDT in root canal disinfection. The available studies showed a positive effect of this therapy in the reduction of microbial load in root canal treatment. However, aPDT promotes some harmful effects to cells and the extent of cell damage depends on some parameters. The present study applied a specific system for aPDT use in endodontics, using a three-dimensional laser through a flexible optical fiber tip throughout the root canal length. The root canals were conditioned with a phenothiazine-based photosensitizer at 10 mg/mL concentration in a specific system for use in the root canal system (Helbo Blue). Although aPDT stimulated angiogenesis and the expression of bone formation markers, its results were worse than those obtained with two-session endodontic treatment using a CH-based dressing. Therefore, additional studies should be conducted to establish the most adequate parameters for the safe use of aPDT in teeth with pulp necrosis and apical periodontitis.

### Table 3. Data of the immunohistochemical analysis of OPN – percentage of each score in each group and p-value.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Score 0</th>
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Conclusion

Although endodontic treatment in one-session supplemented with aPDT stimulated angiogenesis and the expression of bone formation markers, the two-session endodontic treatment with a CH-based dressing stimulated these processes more intensely and promoted better apical periodontitis repair.

References


