Effect of epigallocatechin-3-gallate (EGCG) - based paste as intracanal dressing, in matrix metalloproteinases 2 and 9 in dog’s periapical lesions

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Abstract

Objectives: High expression of MMP-2 and MMP-9 in periapical lesions plays an important role in the degradation of the extracellular matrix. This study aimed to investigate the effect of epigallocatechin-3-gallate (EGCG)-based endodontic paste as an intracanal dressing on the expression of MMP-2 and MMP-9 in periapical lesions.

Materials and Methodss: Periapical lesions were experimentally induced in 35 mature beagle dog premolars randomly divided into healthy teeth, untreated periapical lesions, periapical lesionstreated in a single session, and periapical lesions treated in two sessions with EGCG or calcium hydroxide-based pastes. The operator monitored the animals and performed euthanasia after 120 days for histopathologic and immunofluorescence analyses to assess the expression of MMP-2 and MMP-9. Then we perform the statistical analysis.

Results: Endodontic treatment in two sessions using EGCG and calcium hydroxide-based pastes provided similar levels of repair of the apical and periapical tissues and neoformation of periodontal ligament fibers, cementum and alveolar bone. In both groups, the expression of MMP-2 and MMP-9 was minimal, and it was observed in the cytoplasm of fibroblasts, osteoblasts, cementoblasts, cementocytes and vascular endothelium. In healthy teeth, the expression of MMPs was minimal and was found in odontoblasts. Endodontic treatment in a single session did not result in full repair of periapical lesions, and they presented intense expression of MMP-2 and MMP-9, including in the cytoplasm of persistent inflammatory cells, similar to untreated lesions. In both groups treated in two sessions, the expression of MMP-2 and MMP-9 was similar to that in healthy teeth, and it was significantly lower than that in periapical lesions treated in a single session or untreated (p <0.001).

Conclusions: The use of EGCG-based endodontic paste reduced the expression of MMP-2 and MMP-9 and allowed for the repair of periapical lesions, similar to calcium hydroxide-based paste, and it was superior to treatment performed in a single session.

Clinical Relevance: This study was the first one that evaluated the use of EGCG-based paste as intracanal treatment resulted in a reduction of the expression of MMPs 2 and 9 and repair of periapical lesions, similarly to calcium hydroxide-based paste.

Introduction

Matrix metalloproteinases (MMPs) are a family of more than 25 zinc- and calcium-dependent proteases related to the function and structure of tissues. They are mainly involved in the degradation of protein components of the extracellular matrix (ECM), including dentin and bone [1]. MMPs are produced by polymorphonuclear leukocytes, keratinocytes, monocytes, macrophages, fibroblasts and mesenchymal cells, and they may be present inside inflammatory cells [2]. MMPs are most often isolated on the cell surface or inside the extracellular matrix [3], or they are synthesized as inactive zymogens, which can
occur through intracellular, extracellular or cell surface-mediated proteolytic mechanisms [4]. MMP expression in healthy tissues is present at low levels; however, in inflammatory and infectious pathological conditions such as periodontal and periapical diseases, MMP levels are elevated [3–5].

During the progression of pulpal and periapical diseases, MMP-2 and MMP-9 play an important role in the immune and inflammatory response of the host, promoting the degradation of the extracellular matrix and the maintenance of pulp tissue and periapical inflammation [4]. High expression of MMP-2 and MMP-9 can be found in periapical lesions and intracanal exudates of teeth with acute periapical abscesses, in periapical granulomas and cysts and in crevicular gingival fluid of teeth with chronic periapical lesions [3, 5]. The increase in MMP expression during periapical lesion development can be reduced by endodontic treatment performed with the use of calcium hydroxide intracanal medication but not with endodontic treatment performed in a single visit [5].

Aiming to reduce toxicity and microbial resistance caused by synthetic drugs, herbal extracts have been proposed in the prevention and treatment of autoimmune, infectious and inflammatory diseases [6–9]. The Green tea, derived from *Camellia sinensis*, has been widely studied due to its beneficial effects on general and oral health [7–9]. These effects are due to the presence of epigallocatechin-3-gallate (EGCG), which has proven benefits as an antioxidant, anti-inflammatory [10], anti-carcinogenic [11], antihypertensive [12] and regenerative agent on soft and hard tissues [13]. In addition, green tea has antimicrobial activity [6, 14], presenting a high spectrum of action against Gram-positive and Gram-negative bacteria [15], as well as having antifungal properties [16].

In endodontics, the use of EGCG has been suggested as a medication between sessions [17] or as an irrigant of root canals [18]. Due to its strong antioxidant properties, the use of EGCG as a final irrigant may neutralize any residual sodium hypochlorite and oxygen and increase the bond strength of root canal sealers [19] and bonding systems to root dentin [20]. Recently, studies carried out by our research group developed a formulation based on EGCG for endodontic use that presented tissue compatibility and promoted the healing of periapical tissues [17], and the treatment of post radiotherapy teeth with EGCG inactivates the enzymatic activity of MMPs activated by radiotherapy [21]. Thus, it appears that in addition to playing an anti-inflammatory, antioxidant, antimicrobial and tissue regenerator role, intracanal medication with EGCG may inactivate MMPs. However, the effects of EGCG-based paste on the reduction of MMP-2 and MMP-9 in AP were not suggested or demonstrated *in vivo* after topical use. Thus, the aim of this study was to investigate the effect of intracanal dressing epigallocatechin-3-gallate (EGCG)-based paste on the expression of MMP-2 and MMP-9 during the repair of periapical lesions.

**Materials And Methods**

**Formulation composition**

Our study used EGCG derived from green tea and available on 5 µm solids provided in the solid-state (E41430; Sigma–Aldrich; St Louis, MO, USA). We prepared the EGCG-based paste for endodontic use as
an intracanal dressing as previously reported [16] at a 1 mg/mL concentration using polyethylene glycol 400 (PEG 400, Galena Química e Farmacêutica Ltda., Campinas, SP, Brazil) as a vehicle and zinc oxide (2 g) (SS White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil) as a radio-opacity agent. We used calcium hydroxide paste (Calen®, S.S. White Dental Articles Ltda.) as a control because it is currently the most common medication used during endodontic treatment. Calen paste is comprised of 2.5 g calcium hydroxide and 0.05 g colophony, with 0.5 g zinc oxide as a radio-opacity agent and 2 mL polyethylene glycol 400 (PEG 400) as a vehicle.

**Animals**

All animal procedures in this study were performed while conforming to protocols reviewed and approved by the Animal Care Committee of the University of São Paulo (Protocol #11.1.1405.53.8). The studies also followed standards recommended by the International Organization for Standardization (ISO) n° 7405/2008 (dogs), with the exception of the recommended experimental periods (28 and 90 days), aiming to restrict the number of animals to the minimum necessary to obtain conclusive results, in accordance with the current ARRIVE (Animal Research: Reporting of In Vivo Experiments).

Laboratory Animal Care Facility at the Medical School of Ribeirão Preto, Brazil, gave in three beagle dogs of both genders at 12 months of age and 33 pounds. For the experiment, we selected the 2nd and 3rd upper premolars and the 2nd, 3rd and 4th lower premolars, totaling 35 teeth (70 roots). In cases of gingivitis and/or gingival calculus, the animals received prophylaxis, scraping, straightening and dental polishing, followed by the application of 0.12% chlorhexidine digluconate (Periogard - Colgate - Palmolive - Indústria Ltda. - Brazil).

**Operative procedures**

The same operator performed all procedures. He preanesthetized and subsequently anesthetized the animals intravenously with zolazepam. For induction of periapical lesions after crown access, the operator removed the pulp tissue and left the root canals exposed to the oral cavity for 7 days to allow microbial contamination [22]. Afterward, he sealed the cavities with zinc oxide-eugenol cement (IRM®, Dentsply Industria e Comércio LTDA - Petrópolis - Brazil). Before the operative procedures, the operator took standardized periapical radiographs until the development of periapical radioluencies, which occurred after 45 days.

After this period, the teeth were isolated with a rubber dam and disinfection of the operative field was made with 2% chlorhexidine gluconate. The working length was determined to be 1 mm short of the radiographic apex and confirmed by xRay periapical radiography. Apical delta was perforated by using #20 to #25 K-files at the length of the tooth, thus creating a standardized apical opening. The root canals were instrumented by ProTaper Universal rotary system (DentsplyMaillefer, Ballaigues, Switzerland) in the sequence recommended by the manufacturer (S1 0.18/.02, S2 0.20/.04, F1 0.20/.07, F2 0.25/.08, F3 0.30/.09, F4 0.40/.06 and F5 0.50/.05) using the XSmart™ endodontic micromotor (Dentsply Maillefer Instruments; Ballaigues, Switzerland) under irrigation with 3.6 mL 2.5% NaOCl at each file change. The ethylene-diamine-tetra-acetic acid (EDTA) solution was used as a penultimate wash for 3 minutes under
agitation with a k file followed by final rinse with NaOCl solution. Five groups were formed according to the following experimental conditions (Table 1):

<table>
<thead>
<tr>
<th>Clinical conditions and treatments</th>
<th>Number of teeth (roots)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy tooth</td>
<td>5 teeth (10 roots)</td>
</tr>
<tr>
<td>Tooth with untreated periapical lesion</td>
<td>5 teeth (10 roots)</td>
</tr>
<tr>
<td>Tooth treated endodontically in a single session</td>
<td>5 teeth (10 roots)</td>
</tr>
<tr>
<td>Tooth treated endodontically in two sessions: paste based on EGCG</td>
<td>10 teeth (20 roots)</td>
</tr>
<tr>
<td>Tooth treated endodontically in two sessions: paste based on calcium hydroxide</td>
<td>10 teeth (20 roots)</td>
</tr>
</tbody>
</table>

Healthy group (healthy and nontreated teeth): MMP-2 and MMP-9 expression in healthy tissues was characterized.

Untreated group (teeth with untreated periapical lesions): MMP-2 and MMP-9 expression in periapical lesions experimentally induced and nontreated endodontically was characterized.

One session group (teeth with induced periapical lesions submitted to endodontic treatment performed in a single session): the root canal filling was finished in the same session after chemomechanical preparation.

EGCG group (teeth with induced periapical lesions submitted to endodontic treatment performed in two sessions with EGCG-based intracanal dressing): root canal filling was performed 14 days after the application of endodontic medication.

Calcium hydroxide group (teeth with induced periapical lesions submitted to endodontic treatment performed in two sessions with calcium hydroxide-based intracanal dressing): The root canal filling was applied 14 days after the application of endodontic medication.

In the EGCG and calcium hydroxide groups, the operator applied an intracanal dressing with each material 1 mm beyond the working length to promote a very slow extrusion of the medication into the periradicular tissues, which was radiographically assessed. This procedure was performed with the aid of an ML threaded syringe (S.S. White Artigos Dentários Ltda.; Rio de Janeiro, Brazil) and a long needle 27G (Septoject XL; Septodont, France). Sealing was achieved with glass-ionomer-based cement for 14 days. At the end of this period, the operator removed intracanal dressing by irrigation and performed root filling.

To carry out the root filling, we used AH Plus sealer (De Trey; Dentsply, Konstanz, Germany) and gutta-percha cones by lateral condensation with a final radiographic confirmation. Then, the operator restored
all teeth with a base of glass–ionomer cement and silver amalgam.

**Histotechnique processing**

After 120 days of the first section of endodontic treatment, all animals were euthanized. Tooth presenting extruded filling was excluded from study. The maxilla and mandible were removed, dissected, sectioned, fixed, washed and subjected to decalcification. Subsequently, the pieces were neutralized, washed, dehydrated in alcohol, cleared in xylol and embedded in paraffin. Serial longitudinal sections 5µm-thick were cut in mesiodistal orientation. For histopathological analysis, the sections were initially stained with hematoxylin and eosin (H&E), and evaluated by conventional light microscopy (Leica DMR, Leica Microsystems Wetzlar Gmbh; Wetzlar, Germany).

Microscopic analysis was performed by two examiners, with the Kappa (K) concordance test at k = 0.81 without prior knowledge of the group to which the analyzed specimen belongs. They evaluated the integrity of the extracellular matrix and the presence and intensity of the inflammatory infiltrate on slides stained with H&E under conventional and fluorescent light microscopy by means of quantitative analysis. An assessment of the degree of tissue disorganization was carried out to characterize the repair stage through the reinsertion and neoformation of the fibers of the periodontal ligament attached to the apical third. We classify the degree of collagen fiber disruption and disorganization as (1) absent, (2) minimal, (3) moderate or (4) severe to indicate the level of apical periodontal ligament destruction. We use this same criterion to assess the integrity of the adjacent cementum and alveolar bone.

**Immunofluorescence processing**

In order to evaluate the expression of matrix metalloproteinases and the distribution of these enzymes in tissues (pulpal, apical and periapical region), immunofluorescence assays were performed for MMP-2 and MMP-9.

The slides were deparaffinized, hydrated in a decreasing series of alcohols, and washed under running water. The antigenic recovery with Proteinase K 1: 500 (Invitrogen, Carlsbad, USA) performed for 10 minutes. The slides were washed in phosphate buffered saline (PBS) for 5 minutes (3 times). After that, sodium borohydride 1mg / mL (Dinâmica Química Contemporânea LTDA, Indaiatuba, Brazil) was applied for 15 minutes (3 x). Washed again in PBS for 5 minutes (3 x) and the nonspecific binding sites blocked with 1% bovine serum albumin (Sigma, St Louis, USA) for 60 minutes. The tissues were incubated with the primary antibodies in 1:50 concentration for MMP-2 (5 g / mL; 53630, Santa Cruz Biotechnology, Dallas, USA) and MMP-9 (5 g / mL; 21736, Santa Cruz Biotechnology) at 4 ºC overnight. Then, the slides were removed from the refrigerator and placed at room temperature for 1 hour. After that, they were washed in PBS for 5 minutes (3 times). The slides were then incubated with biotinylated secondary anti-mouse FITC antibodies at a concentration of 1: 200 for 1 hour (Rabbit anti-mouse IgG-FITC sc-358916, Santa Cruz Biotechnology), washed in PBS for 5 minutes (3 times), and finally, the coverslips were placed using UltraCruz® Aqueous Mounting Medium with DAPI (24941, Santa Cruz Biotechnology, Dallas, USA). Control slides were used to test the specificity of the immunostaining in which the primary antibody was omitted and the slides were incubated in phosphate buffered saline (PBS).
The microscopic analysis was performed by two examiners with the Kappa (K) concordance test at k = 0.83, without prior knowledge of the group to which the analyzed specimen belongs.

**Statistical analysis**

The quantitative histopathological results (tissue disorganization, inflammatory infiltrate and MMP-2 and MMP-9 expression) are ordinal, independent variables with three different categories and sizes for the experimental and control groups. Thus, for each variable, the Kruskal-Wallis nonparametric test was used, followed by Dunn’s post-test for multiple comparisons. The statistical software used was SPSS version 25 (IBM, Chicago, USA) with a significance level of 5% (p < 0.05).

**Results**

**Descriptive Histological Analysis**

In Healthy Group, the intact premolars presented pulp vitality in the coronary and root regions, integrity of the apical and periapical tissues and absence of inflammatory infiltrate (Fig. 1.A and B). Positive and minimal expression of MMP-2 and MMP-9 (score 2) was observed, located in the fibroblast membrane and cytoplasm between the fibers of the periodontal ligament (Fig. 2.A), cementoblasts and osteoblasts lining cement and bone surface. MMP-9 expression in osteoblasts was present in mature bone, with evidence of the transition process from newly formed bone and lamellar bone during the physiological process of bone remodeling (Fig. 2.B).

The teeth of Untreated Group, where induction of periapical lesions was not followed by endodontic treatment, presented pulp necrosis in the entire length of the root canal associated to the presence of a chronic inflammatory infiltrate, composed of polymorphonuclear and mononuclear cells, in the apical and periapical region, associated to resorption of the fibers of the periodontal ligament, resorption of external and internal surface of cementum with absence of cementocytes in some lacunaes. The resorption of the alveolar bone surrounding the periapical lesion was also observed (Fig. 1.C and D). There was a positive and intense immunostaining of MMP-2 and MMP-9 in the periapical lesion, located inside the cementocyte’s lacunae and in the cells of the inflammatory infiltrate and cementoblasts (Figs. 2. C-D).

After endodontic treatment performed in one-session Group, without the application of endodontic dressing, the persistence of a severe inflammatory infiltrate and resorption of mineralized tissues, including cementum and alveolar bone and absence of repair of periodontal ligament fibers was observed (Fig. 1.E-F). There was a positive and intense expression of MMP-2 and MMP-9 in the periapical lesion, similar to teeth with untreated periapical lesion (p > 0.05). Expression of MMP-2 and MMP-9 can be seen in the cells of the inflammatory infiltrate throughout the periapical lesion and inside the cementocyte lacunae still resorbed as consequence of the inflammatory response (Fig. 2.E-F).

In EGCG Group, the teeth treated in two sessions by the use of EGCG dressing prior root canal filling, it was possible to visualize the repair of periapical lesions, with reestablishment of the periodontal ligament
space with reinsertion of collagen fibers, bone and cement neoformation, and absence of inflammatory infiltrate (Fig. 1.G-H). In this group, a minimal expression of MMP-2 and MMP-9 was observed in the periapical region, similar to healthy tissues (Fig. 2.G-H). A significant reduction of the immunofluorescent expression of MMP-2 and MMP-9 was observed after endodontic treatment using an EGCG-based paste compared to the expression of MMP-2 and MMP-9 in the teeth with untreated periapical lesions or treated in one-session (p < 0.05).

In Calcium Hydroxide Group, it was also possible to observe the repair process of periapical lesions, with reinsertion of collagen fibers at periodontal ligament, neoformation of alveolar bone and cementum at external and internal root surface and absence of inflammatory infiltrate (Fig. 1.I-J). A positive and minimal expression of MMP-2 and MMP-9 was observed, similar to the group treated with EGCG-based paste (p > 0.05), with significant reduction of its immunofluorescent expression when compared to teeth of Untreated and One-session Groups (p < 0.001). It was also possible to visualize the immunostaining of MMP-2 and MMP-9 in cells such as odontoblasts and cementoblasts, which configure the participation of these proteases in physiological processes of bone tissue repair and tissue repair of the root cementum (Fig. 2.I and 2.J).

**Quantitative analysis by scores**

The results of the quantitative histological analyzes (tissue disorganization, inflammatory infiltrate and expression of MMP-2 and MMP-9) are listed in Table 2.
Table 2
Percentage of roots (n) for each score, according to a histological analysis performed by different clinical conditions and after different protocols of endodontic treatment.

<table>
<thead>
<tr>
<th>Quantitative histological parameters</th>
<th>Scores</th>
<th>Clinical conditions and treatments</th>
<th>Healthy Tooth</th>
<th>Untreated Periapical Lesion</th>
<th>Single Session</th>
<th>EGCG-based paste</th>
<th>Calcium hydroxide based paste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue disorganization</td>
<td>Absent</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td></td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td></td>
<td>0</td>
<td>100</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td></td>
<td></td>
<td><strong>b</strong></td>
<td><strong>a</strong></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>b</strong></td>
</tr>
<tr>
<td>Inflammatory Infiltrate</td>
<td>Absent</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>10</td>
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<tr>
<td></td>
<td>Moderate</td>
<td></td>
<td>0</td>
<td>0</td>
<td>30</td>
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<td>0</td>
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<tr>
<td></td>
<td>Severe</td>
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<td>0</td>
<td>100</td>
<td>70</td>
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<td>Statistical analysis</td>
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<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>b</strong></td>
</tr>
<tr>
<td>Expression of MMP-2</td>
<td>Absent</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>Minimal</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
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<td>0</td>
<td>0</td>
<td>40</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td></td>
<td>0</td>
<td>100</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td></td>
<td></td>
<td><strong>b</strong></td>
<td><strong>a</strong></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>b</strong></td>
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<tr>
<td>Expression of MMP-9</td>
<td>Absent</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>Minimal</td>
<td></td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>85</td>
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<tr>
<td></td>
<td>Moderate</td>
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<td>10</td>
<td>0</td>
<td>30</td>
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<td>15</td>
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<tr>
<td></td>
<td>Severe</td>
<td></td>
<td>0</td>
<td>100</td>
<td>70</td>
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<tr>
<td>Statistical analysis</td>
<td></td>
<td></td>
<td><strong>b</strong></td>
<td><strong>a</strong></td>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
<td><strong>b</strong></td>
</tr>
</tbody>
</table>

The Kruskal–Wallis nonparametric test and Dunn’s posttest were used. Different letters indicate significant differences (p < 0.05).
There were no statistically significant differences between untreated or single visit treated teeth (p > 0.05), which presented severe scores in most of the specimens for all parameters evaluated: tissue disorganization, inflammatory infiltrate and expression of MMP-2 and MMP-9. There were also no differences between periapical lesions treated with EGCG and calcium hydroxide (p > 0.05) that presented most of the specimens with absence of tissue disorganization and inflammatory cells infiltration and minimal for expression of MMP-2 and MMP-9, similarly to healthy teeth (p > 0.05). Teeth with untreated periapical lesions or with periapical lesions treated in a single session were different from teeth treated in two sessions with EGCG or calcium hydroxide and healthy teeth (p < 0.01).

Discussion

The present study, that had as objective to evaluate the effect of EGCG endodontic application on MMPs 2 and 9 expression in periapical lesions, shows under histopathological analysis, that the EGCG- and the calcium hydroxide-based endodontic pastes provided the repair of the apical and periapical tissues, with reestablishment of the space of the periodontal ligament, neoformation of cementum and alveolar bone and absence of inflammation. The occurrence of healing of apical periodontitis after the use of calcium hydroxide-based paste has been proven in several studies [6, 22–25]. However, in the analysis by means of immunofluorescence, the present study shows for the first time, that the use of EGCG and calcium hydroxide, similarly promote reduction of the expression of MMP-2 and MMP-9 in periapical lesions, in levels similar to healthy teeth.

The expression of MMPs have being found in association with the Toll-like receptors 2 and 4 in symptomatic and asymptomatic apical periodontitis, what may explain the clinical presentations and the evolution of apical periodontitis and may represent key targets for new diagnostic and treatment approaches [26]. Furthermore, the present study allowed the identification of a positive staining for MMPs 2 and 9 in cells such as odontoblasts, osteoblasts, cementoblasts, cementocytes, fibroblasts and blood vessels, which shows the important participation of these enzymes in tissue repair processes of apical periodontitis. It was reported that EGCG inhibited the formation and differentiation of osteoclasts via inhibition of MMPs 2 and 9 in rats [27]. In addition, EGCG could prevent the alveolar bone resorption that occurs in periodontal diseases, by inhibiting the expression of MMP-9 in osteoblasts and the formation of osteoclasts [28]. Other studies also show the immunostaining of MMP-2 and MMP-9 in osteoblasts, however, in the respective studies, rats and mice were used as an experimental animal model [29]. In the present study we use beagle dogs as model for histopathological study of apical periodontitis due its anatomical and physiopathological similarities with humans. Experimental induction of apical periodontitis in dogs teeth has being performed by histopathological studies for evaluation of new endodontic materials and modalities of treatment under the same operatory conditions applied in humans and with healing responses obtained in shorter periods than humans [30].

The present study also showed that teeth endodontically treated in a single session presented intense expression of MMP-2 and MMP-9 2 in the periapical region, persistence of inflammatory infiltration and tissue disorganization and resorption, similar to periapical lesions experimentally induced and not
submitted to endodontic treatment. The expression of MMP-9 and transforming growth factor beta (TGF-β1) was previously evaluated in samples of periapical lesions and correlated with the intensity of the inflammatory infiltrate and the thickness of the epithelial lining, showing that the extracellular matrix remodeling process is dependent of MMP-9 appears to be similar for periapical granulomas and root cysts [24]. MMP-9 and TGF β1 can play an important role in maintaining periapical injuries [31]. In addition, MMP-2 and MMP-9 play a critical role in the development of periapical inflammatory lesions, probably involved in the degradation of the extracellular matrix (ECM) during the early stage of development of periapical injury in rats [5]. In a similar study, microbiologic findings showed that teeth treated with calcium hydroxide root canal dressing exhibited a lower percentage of bacterial contamination, a lower MMP expression, and a more organized extracellular matrix, unlike those treated in a single visit. Our study had the difference of being carried out after 120 days and not 180, aiming to reduce the period of animal experimentation since the radiographic follow-up have already shown conclusive results of periapical lesion repair at 120 days.

The differences in the histopathological repair of periapical lesions observed between teeth endodontically treated in a single session or two sessions may be attributed to differences in the reduction of intra- and extraradicular infection. It is well known that the use of an endodontic medication that presents tissue compatibility, including its intentionally slow extrusion into the periradicular tissues, may improve eradication of root canal system infection, especially extraradicular infection and periapical biofilms, that may not be affected by chemomechanical procedures, and such persistence is associated with failures of endodontic treatment and the maintenance of lesions [2].

A factor that may also be involved with the observed results is related to the two sessions of endodontic treatment, which allowed for the use of more irrigation and instrumentation during the second visit, which may also favor resolution of the infection present inside the root canal. The irrigation method may interfere with the reduction of intraradicular endodontic infection. In the present study, we used conventional irrigation, but previous in vivo histopathological studies have suggested better revascularization and periapical repair after endodontic treatment using apical negative pressure irrigation versus conventional irrigation intracanal dressing in dogs' immature teeth [33] and mature teeth with apical periodontitis [34]. Thus, considering that intra- and extraradicular biofilm removal and inactivation may be accomplished by both chemomechanical processes and intracanal medicaments, further clinical, radiographic, microbiologic and histopathological studies should be performed to assess the repair of periapical lesions using different irrigation activation systems performed in one or two sessions and associated or not with intracanal medications with EGCG or calcium hydroxide.

This study was the first to evaluate the use of EGCG-based paste as an intracanal treatment, which resulted in a reduction in the expression of MMPs 2 and 9 and repair of periapical lesions, similar to calcium hydroxide-based paste. A point to be considered is that the pure form of EGCG, extracted from the Camellia sinensis plant used in the present study, is commercialized in powder form and needs to be prepared by adding vehicle and radio-opacity before use. Therefore, a limitation of using EGCG-based paste for endodontic use is its high costs in comparison to calcium hydroxide and its non-existence on
the market since there is still no commercially available EGCG endodontic formulation. On the other hand, in addition to presenting tissue compatibility, based on its antimicrobial action against several species involved in endodontic infection and inhibition of the proteolytic activity of MMPs, the antioxidant role of EGCG presents double effects on neutralizing the side effects of sodium hypochlorite and increasing bond stability and adhesion filling sealers [19, 20]. Thus, additional studies are required to evaluate the extensive properties of EGCG, including its potential to act as a biomodifier of the internal root canal surface and its involvement in other signaling pathways or tissue inhibitors of MMPs 2 and 9, which can be evaluated with different methodologies that can be applied during endodontic treatment and repair of mineralized and nonmineralized tissues.

**Conclusions**

It can be concluded that the EGCG-based paste used as an intracanal dressing in Beagle dogs resulted in reduction of the immunofluorescent expression of MMP-2 and MMP-9, and the histopathologic repair of periapical lesions, similarly to the calcium hydroxide-based paste and superior to the treatment of teeth with periapical lesion performed in a single session. The expression of MMPs 2 and 9 was found in odontoblasts, osteoblasts, cementoblasts, inflammatory infiltrate cells, blood vessels, fibroblasts and cementocytes.

**Declarations**

**Ethical Approval**

All animal procedures in this study were performed while conforming to protocols reviewed and approved by the Animal Care Committee of the University of São Paulo (Protocol #11.1.1405.53.8).

**Competing interests**

The authors deny any conflicts of interest.

**Authors’ contributions**

A.R., T.P. and J.L. wrote the main manuscript text. All authors reviewed the manuscript.

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**Availability of data and materials**

Not applicable
References


**Figures**
Figure 1

Representative microscopic images of dogs apical and periapical tissues stained with HE and evaluated under conventional light microscopy (left images) and fluorescence microscopy (right images). A and B: healthy tooth, C and D: tooth with untreated periapical lesions; E and F: periapical lesions endodontically treated in a single session; G and H: periapical lesions endodontically treated in two sessions with EGCG-
based paste and I and J: periapical lesions endodontically treated in two sessions with calcium hydroxide-based paste. Magnification 5X.

Figure 2

Representative microscopic images from immunofluorescence staining of MMP-2 (left images) and MMP-9 (right images) in dogs apical and periapical tissues. A and B: healthy tooth showing positive and
minimal expression of MMP-2 in membrane and cytoplasm of fibroblasts at the periodontal ligament space, cementoblasts and osteoblasts, and MMP-9 in osteoblasts present in mature bone. C and D: tooth with untreated periapical lesions presenting positive and intense immunostaining of MMP-2 and MMP-9 in cementocyte lacunae, inflammatory infiltrate cells and cementoblasts. E and F: periapical lesions endodontically treated in a single session presented intense expression of MMP-2 and MMP-9 in persistent cells of inflammatory infiltrate and cementocyte lacunae. G and H: periapical lesions treated with EGCG-based paste presented minimal expression of MMP-2 and MMP-9 located in cementocytes, cementoblasts and osteoblasts lining repaired alveolar bone surface. I and J: periapical lesions treated with calcium hydroxide-based paste showed a positive and minimal expression of MMP-2 and MMP-9 in cementoblasts and fibroblasts. Fluorescence microscopy. Magnification: A, C-F: 10X; B 20X; G-J: 40X.