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ORIGINAL ARTICLE

Apical Periodontitis Healing Following Treatment is Impacted by Root Canal Sealer Composition: An *in Vivo* and *in Vitro* Investigation

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ABSTRACT

Objective: To evaluate the periapical healing following root canal treatment in teeth with apical periodontitis (*in vivo*) and the cytotoxic potential of root canal sealers *in vitro*. **Material and Methods:** Apical periodontitis was induced in 60 dogs' teeth and root canals were filled with Sealapex (40 roots), EndoREZ (40 roots), intracanal dressing (20 roots), or left untreated (20 roots). After 30 and 90 days, histopathological analyses were made. *In vitro*, J774.1 macrophages were stimulated with root canal sealers extracts, cytotoxicity was assessed using lactate dehydrogenase assay, and qRT-PCR was used to analyze TNF-α gene expression. **Results:** *In vivo*, smaller apical periodontitis and lower inflammatory cell infiltrate were found in teeth treated with Sealapex compared to EndoREZ. *In vitro*, EndoREZ was cytotoxic and induced TNF-α gene expression by macrophages differently from Sealapex. **Conclusion:** Sealapex allowed improved tissue repair following root canal treatment in teeth with apical periodontitis compared to EndoREZ. Synthesis of TNF-α induced by LPS was enhanced by EndoREZ, whereas Sealapex prevented pro-inflammatory gene expression.

Keywords: Endodontics; Root Canal Obturation; Periapical Diseases.

Introduction

A major challenge in dentistry is the treatment of root canals in teeth with pulp necrosis and apical periodontitis. This treatment aims to reduce the infection present in the root canal system [1,2]. Root canal obturation is the final stage and must be done with biocompatible materials since they will remain in contact with apical tissue for a long period [3,4]. Currently, it is made with a solid material associated with a sealer for perfect and three-dimensional sealing. Gutta-percha is a solid material and has been widely used because of its beneficial properties [5].

Currently, endodontic sealers are categorized by composition based on setting reaction and composition: zinc oxide eugenol, salicylate, glass ionomer, silicone, epoxy resin, bioceramics, and methacrylate resin sealer system. Root canal sealers should present biocompatibility and antibacterial effects, along with the ability to produce a hermetical seal, dimensional stability and radiopacity [6]. However, none of the sealers commercially available present all properties of an ideal material [7-9]. EndoREZ is a type of resin sealer, which has hydrophilic properties. Studies show that this material presents sealing ability in the presence of moisture and penetration in the dentin tubules, in addition to moderate *in vitro* cytotoxicity [10-12]. Sealapex is a root canal filling that contains calcium hydroxide in its formula and its biological properties have been extensively investigated [13,14]. Nonetheless, the influence of those materials on periapical healing following root canal treatment in teeth with necrosis and apical periodontitis is not well known.

Considering the importance of root canal filling for successful root canal treatment, it is important to evaluate the performance of root canal sealers in teeth with pulp necrosis and apical periodontitis. Therefore, this study aimed to investigate the periapical healing following root canal treatment in teeth with apical periodontitis using EndoREZ and Sealapex sealers and the cytotoxicity of both root canal sealers *in vitro*. The null hypothesis of this study was that EndoREZ and Sealapex sealers did not present toxicity *in vitro* and *in vivo*.

Material and Methods

In vivo Study

This study was initially submitted and approved by the Animal Care Committee of the University of São Paulo (Protocol #11.1.1405.53.8). The methodology and sample size were based on the protocol recommended by the International Organization for Standardization standard Biological Evaluation of Dental Materials (ISO 7405:2008) [15]. Furthermore, the animal experiments in this study are in accordance with the current ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines [16].

Before the operative procedures, the animals received antihelminthic medication (Drontal Puppy, Bayer S. A., São Paulo, SP, Brazil), vitamins (Glicopan Pet, Vetnil Ind. Com. Produtos Veterinários Ltda., Louveira, SP, Brazil) and vaccines, in 3 doses, with an interval of 3 weeks between each application. During the entire experimental period, dogs were kept in the vivarium of the University with free access to water and a standard diet.

For root canal therapy, the animals were pre-anesthetized by means of intravenous injection of 1mg/kg of Neozine (Sanofi-Aventis Farmacêutica Ltda., Suzano, SP, Brazil). After sedation, anesthesia was induced with Zoletil 50 (Virbac Brasil, Sorocaba, SP, Brazil) at 0.1 ml/kg administered intravenously to facilitate the passage of the endotracheal tube, necessary to perform inhalation anesthesia. After the intubation, anesthesia was maintained with Isoflurane (Abbott Ltda., Rio de Janeiro, RJ, Brazil). During the entire

operative procedures, the animals were collected with an isotonic 0.9% sodium chloride solution (Glicolabor Ltda., Ribeirão Preto, SP, Brazil).

Eight dogs (12 months of age, weighing from 8 to 15 kg) were submitted to the experimental protocol. The dogs were from the same litter, had no defined breed and they were both genders. Second and third maxillary premolars and the second, third, and fourth mandibular premolars were used. In total, 60 teeth (120 roots) were treated.

Initially, local anesthesia was performed for each quadrant using 2% mepivacaine with noradrenaline 1:100.000 (Scandicaine; Septodont Brasil, Pomerode, SC, Brazil). Then, the crown access was performed and the pulp was removed. Root canals were left exposed to oral cavity for seven days and then were sealed with zinc oxide-eugenol cement (IRM®, Dentsply Ind. Com. Ltda., Petrópolis, RJ, Brazil). The purpose of this step was to generate microbial contamination and to induce apical periodontitis. Standardized radiographs were taken using customized stents. After 45 days, radiolucent periapical images indicating chronic apical periodontitis were observed radiographically [17].

After rubber dam isolation and disinfection (2% chlorhexidine gluconate), interim restorations were removed. The working length was determined to be 1 mm short of the radiographic apex and confirmed by an electronic root apex locator (Root ZXII, Morita Corp., Kyoto, Japan). Apical delta was perforated by means of #20 to #25 K-files to create a standardized apical opening. Instrumentation was carried out using Protaper universal system (Dentsply Maillefer Instruments, Ballaigues, Switzerland) and XSmartTM endodontic micromotor (Dentsply Maillefer Instruments, Ballaigues, Switzerland) under irrigation with 2.5% sodium hypochlorite (1.8 mL) in a syringe (positive pressure) at each file change. The root canals were enlarged from the last rotary instrument to the #70 K-files. The same operator performed all procedures.

The intracanal dressing with calcium-hydroxide-based paste (Calen®; S.S. White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil) was applied to the measurement of 1 mm beyond the working length to promote small extravasation and provide its contact with the external apical surface. This procedure was performed with an ML threaded syringe (S.S. White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil) and a long needle 27G (Septoject XL, Septodont, Saint-Maur-des-Fossés, France). Interim restorations were done with glass-ionomer-based cement for 14 days. At the end of this period, after isolation, the canal dressing was removed and root filling was performed [18].

Next, the procedures were performed according to the technique recommended in each group. The root canals were assigned into eight experimental groups, as follows:

- Groups 1 (30 days; n= 20 dental roots) and 5 (90 days; n= 20 dental roots): The root canals were obturated by using EndoREZ sealer (Ultradent Products, Inc., South Jordan, Utah, EUA) and gutta-percha cones. The EndoRez sealer was manipulated according to the recommendations of the manufacturers. Digital spreader "C" (Dentsply/Maillefer Instruments, Ballaigues, Switzerland) was used in lateral condensation technique. After that, auxiliary gutta-percha points (Dentsply Ind. Com. Ltda., Petrópolis, RJ, Brazil) were used to complete obturation that was confirmed with a final radiographic.
- Groups 2 (30 days; n= 20 dental roots) and 6 (90 days; n= 20 dental roots): Root canals were filled with Sealapex (Sealapex®, SybronEndo Corp., Orange, CA, USA) cement and gutta-percha cones. The filling technique was the same as described for Groups 1 and 5.
- Groups 3 (30 days; n= 10 dental roots) and 7 (90 days; n= 10 dental roots): After performing the biomechanical preparation and using the intracanal dressing, the teeth were filled with calcium hydroxide root canal dressing and restored (negative control).

- Groups 4 (30 days; n= 10 dental roots) and 8 (90 days; n= 10 dental roots): Apical periodontitis healing was induced, but root canal treatment was not done (positive control).

Following root canal treatment, teeth were restored with silver amalgam. After each procedure, postoperative analgesia with tramadol chloridrate was provided and monitoring was performed by a veterinarian doctor at the University of São Paulo.

Histotechnical Processing and Histopathologic Analysis

After the experimental periods of 30 and 90 days, the hemi-arches were radiographed and the animals were euthanized. 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. Specimens were stained in hematoxylin-eosin (HE). Representative sections were stained with hematoxylin and eosin for quantitative analyses under conventional light microscopy as previously described [19,20].

The following parameters were analyzed by a single calibrated examiner (kappa= 0.98): periapical inflammatory infiltrate, apical periodontitis size, presence of bone resorption and apical sealing. For the periapical inflammatory infiltrate, the inflammatory cells were counted per field of view (63x) and for evaluation of apical periodontitis size was used an increasing of 1,25x (mm^2). Both were performed in the HE-stained specimens using the Software Axiovision Rel.4.6 (Carl Zeiss). The sections were observed for determination of scores. Parameters used for analysis were apical sealing with mineralized tissue (present or absent) and bone tissue resorption (present or absent).

In vitro Study

Preparation of Extracts

Experiments were conducted according to International Organization for Standardization [21]. Ten mg of each material were placed on sterile paper and weighed under natural light using a PG 503-S scale (Mettler Toledo, Columbus, OH, USA). Manipulation was performed in a laminar flow cabinet according to the manufacturer's instructions. Next, freshly prepared materials were diluted in 1 mL of Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) for 48 h at 4 °C. A serial dilution was prepared from the initial solution (10 mg/mL) to achieve the final concentration of 1.0 mg/mL [22].

Cell Culture

J774.1 murine macrophage cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in DMEM completed with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After that, cells were reaped with plastic cell scrapers and centrifuged at 1,500 rpm for 10 min at 10 °C using a microcentrifuge. Cells were plated in 96-well culture plates (Corning Glass Works, Corning, NY, USA) at a density of 1×105 cells per well and incubated overnight in DMEM in 5% CO_2 air at 37°C. Next, cell culture media were removed and 200 µl of extracts was added to the wells and plates were incubated for 24h. In a set of experiments, macrophage cells were pre-stimulated with lipopolysaccharide (0.5 mg/mL; Escherichia coli LPS, Sigma-Aldrich, St. Louis, MO, EUA) for two hours. Finally, the medium was removed, and materials were added to the cells.

Cytotoxicity – Lactate Dehydrogenase (LDH) Assay

To evaluate cytotoxicity, the level of LDH released after cell lysis was measured using the CytoTox96® non-radioactive cytotoxicity assay (Promega Corporation, Madison, WI, USA). Absorbance was read by a spectrophotometer at 490 nm (mQuanti, Bio-Tek Instruments, Inc., Winooski, VT, USA). Cytotoxicity index was calculated according to the formula: cytotoxicity (%) = 100 × Experimental LDH Release absorbance / Maximum LDH Release absorbance (positive control).

Real-Time Polymerase Chain Reaction (qRT-PCR)

Total mRNA was extracted using the RNeasy® Mini kit (Qiagen Inc., Valencia, USA) and estimated using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). One µg of total RNA were used for cDNA synthesis (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, USA) in a thermal cycler (Veriti® Thermal Cycler, Applied Biosystems, USA). qRT-PCR reactions to detect Tnf mRNA levels were performed in duplicate using the TaqMan® system (StepOne Plus® Real-Time PCR System, Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and beta-actin (Actb) were used as reference genes for normalization purposes. The cycle program was established as 95 °C for 20 s, 40 cycles at 95 °C for 1 s, and 60 °C for 20 s. Cycle threshold (Ct) values were used as a basis to analyze the results. Relative expression was calculated by the 2-ΔΔCt method.

Statistical Analysis

The quantitative values obtained were evaluated for the type of distribution and compared by means of analysis of variance (ANOVA), followed by the Tukey post-test. The presence / absence of bone resorption and of newly apical sealing were evaluated using Fisher's Exact Test. The significance level was set at 5%.

Results

Inflammatory Cell Recruitment and Apical Periodontitis Healing Following Root Canal Treatment

In teeth that had root canal treatment using EndoREZ or Sealapex, a smaller apical periodontitis lesion was found, both at 30 or 90 days ($p<0.05$). The inflammatory cell infiltrate was also lower in teeth that had root canals treated ($p<0.05$). Apical periodontitis healing at 90 days was more advanced for Sealapex sealer compared to EndoREZ ($p<0.05$). In the group that received intracanal dressing solely (negative control), a smaller apical periodontitis was found ($p<0.05$) with a reduced inflammatory cell number ($p<0.05$) (Figure 1).

Inflammatory cell infiltrate following EndoREZ treatment extended beyond half of the apical periodontal ligament and was predominantly composed of mononucleated cells, with few neutrophils observed, both at 30 and 90 days after root canal filling. Macrophages had sealer particles in their cytoplasm. Areas of apical root resorption were observed and partial biological sealing was found at 90 days after root canals filling (Figure 2; Table 1). Sealapex treatment, on the other hand, resulted in an inflammatory infiltrate composed of mononucleated cells and neutrophils, mostly 30 days after root canal filling, which was reduced at 90 days. Areas of apical root resorption were observed and partial biological sealing was observed in 15% of specimens at 30 days after root canal treatment and in more than 50% of cases at 90 days (Figure 3; Table 1). In the group that received intracanal dressing solely (negative control), at 30 days, the inflammatory infiltrate extended beyond half the apical periodontal ligament and was composed predominantly of mononucleated cells. At 90 days, there was a decrease in the inflammatory cell infiltrate and there was presence of fibers in the periodontal ligament with normal characteristics. Apical root resorption was observed in approximately 30% of cases, with a decrease in prevalence at 90 days, and partial biological sealing was observed in 75% of the specimens at 30

days and in 100% at 90 days. Teeth with apical periodontitis without treatment showed active bone resorption with the recruitment of inflammatory cells and dental root resorption without apical sealing (Table 1).

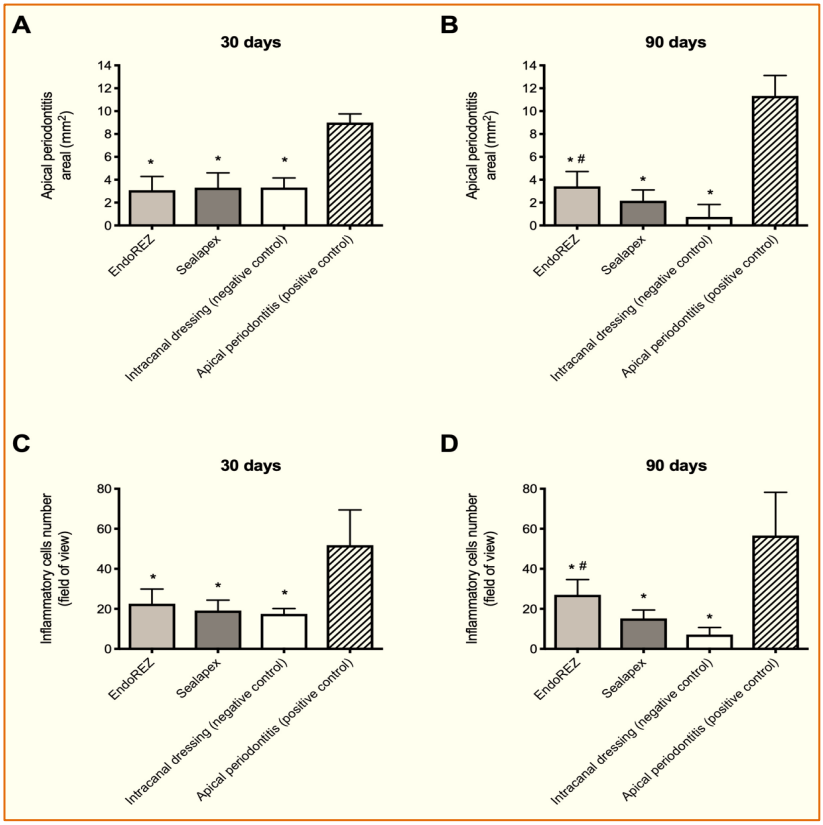

Figure 1. Apical periodontitis area (A, B) and number of inflammatory cells (C, D), 30 and 90 days after root canal treatment with Endo-REZ and Sealapex sealers, calcium hydroxide-based intracanal dressing (negative control) and in teeth with apical periodontitis without treatment (positive control). Graphs depicts mean and standard deviation; *p<0.05 compared to positive control, #p<0.05 compared to negative control.

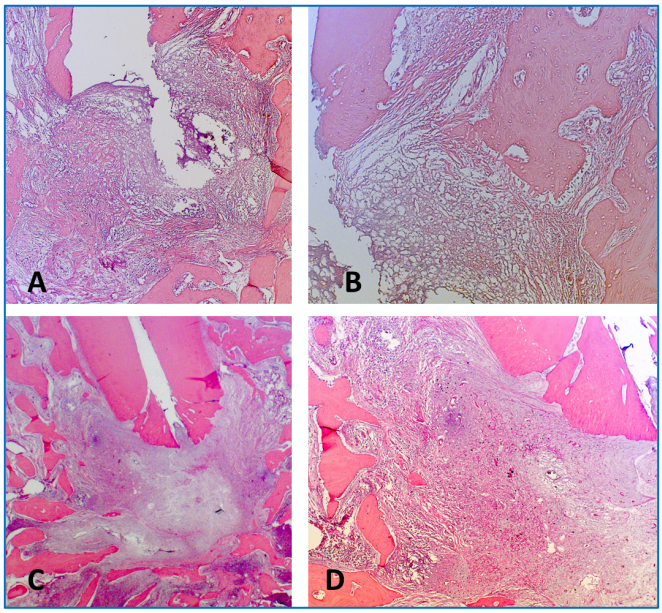

Figure 2. Photomicrographs of representative HE-stained microscopic sections of apical and periapical regions in teeth filled with EndoREZ at 30 (A, B) and 90 (C, D) days.

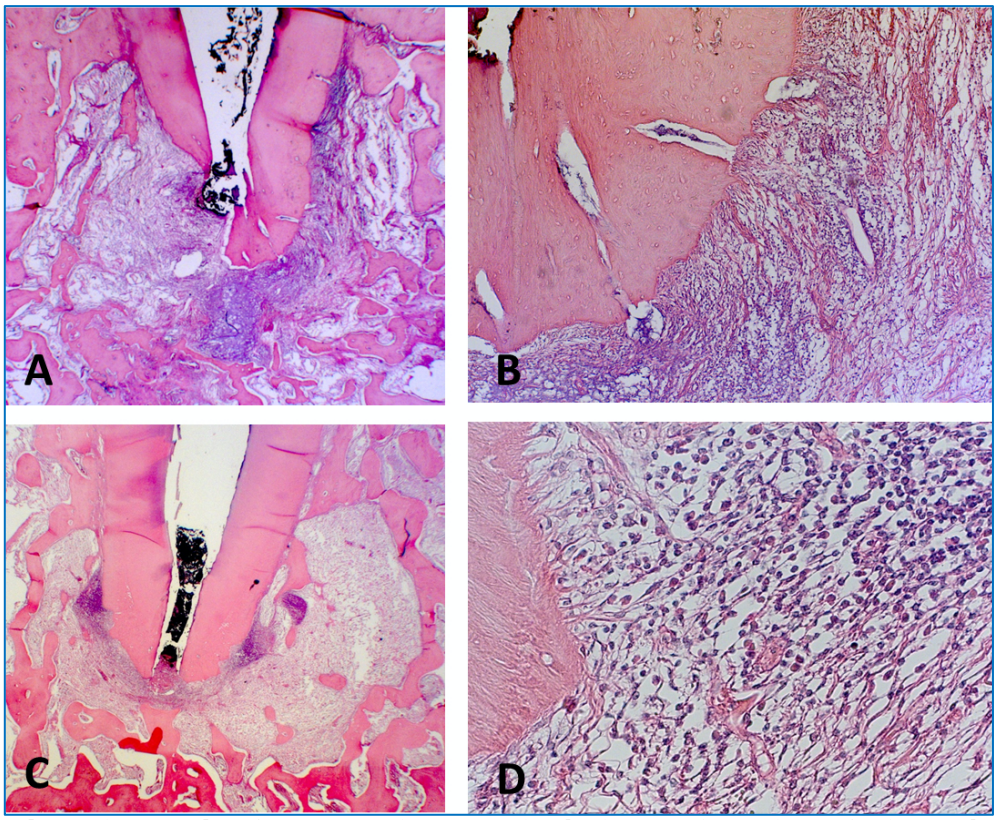

Figure 3. Photomicrographs of representative HE-stained microscopic sections of apical and periapical regions in teeth filled with Sealapex at 30 (A, B) and 90 (C, D) days.

Table 1. Distribution of dental root resorption and apical sealing percentage (%) for each group, according to histopathologic analysis performed 30 and 90 days after root canal filling.

Histopathologic Parameters	Scores	EndoREZ (%)	Sealapex (%)	Intracanal Dressing (%)	Apical Periodontitis without Treatment (%)
Root Resorption (30 Days)	Present	60[a]	28.5[b]	25[b]	100[a]
	Absent	40	71.5	75	0
Root Resorption (90 Days)	Present	70[a]	30[b]	25[b]	100[c]
	Absent	30	70	75	0
Apical Sealing (30 Days)	Present	0[a]	15[b]	75[c]	0[a]
	Absent	100	85	25	100
Apical Sealing (90 Days)	Present	18[a]	50[b]	75[c]	0[d]
	Absent	82	50	25	100

Fisher's Exact Test; Different letters indicate statistical significant differences ($p<0.05$).

Root Canal Sealers Cytotoxicity

Because there was no modulation of periapical inflammation and bone resorption at 30 and 90 days following root canal treatment with EndoREZ, the cytotoxic potential of the sealers was investigated *in vitro*.

EndoREZ root canal sealer induced macrophage cell toxicity (more than 30% of the cells), differently from Sealapex extract ($p<0.05$). When cells were primed with LPS, toxicity was increased for EndoREZ compared to Sealapex or intracanal dressing ($p<0.05$). In addition, Sealapex inhibited the production of TNF-α, whereas EndoREZ induced TNF-α gene expression at 24 h ($p<0.05$). Also, the synthesis of TNF-α induced by LPS was enhanced when EndoREZ was added to cell culture media ($p<0.05$). Sealapex and root canal dressing, on the other hand, prevented TNF-α expression when cells were pre-treated with LPS ($p<0.05$) (Figure 4).

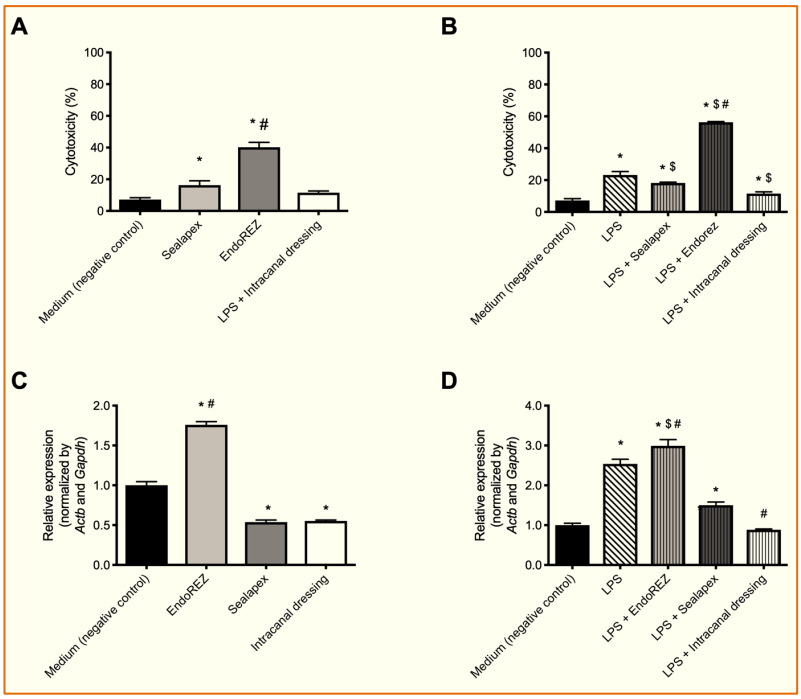

Figure 4. Cytotoxicity (A, B) and TNF-α gene expression (C, D) after stimulation of J774.1 macrophages with root canal sealers EndoREZ and Sealapex, calcium hydroxide-based intracanal dressing or culture medium alone (negative control). In a set of experiments (B, D), J774.1 macrophages were primed with LPS (0.5 mg/mL) prior to root canal sealer stimulation. Graphs depicts mean and standard deviation; * p<0.05 compared to negative control, #p<0.05 comparison between EndoREZ and Sealapex, $ p<0.05 compared to LPS.

Discussion

The null hypothesis of this study was rejected since EndoREZ but not Sealapex sealer present toxicity *in vitro* and *in vivo*. Root canal treatment of teeth with apical periodontitis aims to reduce infection in the apical third, causing repair of the region [23]. Root canal filling is an important step for clinical success of the treatment and the use of root canal sealers that are biocompatible is crucial. Thus, investigations regarding biocompatibility should precede market entrance.

Mineralized sealing of the foraminal aperture is the ideal biological response after root canal treatment [4,14,17,18]. The induction of this sealing is conditioned to the correct execution of all phases of the root canal treatment and also to important factors inherent to these phases, such as the apical limit of obturation and the nature of the obturator material since it will be directly in contact to apical and periapical tissue [24,25]. Both sealers used in this study allowed the deposition of mineralized tissue in the apical third of the root and in the periapical region. This deposition of mineralized tissue was more evident in the Sealapex group at 90 days, compared to EndoREZ group at 90 days. In the negative control group, 100% of cases showed complete sealing at 90 days. These results are similar to those found in the literature that show better results when using Sealapex sealer [14,24,26].

Regarding the intensity and extent of the inflammatory infiltrate, the root canals filled with EndoREZ and Sealapex did not show a reduction in the inflammatory infiltrate over time. However, there was periapical repair when the teeth were maintained only with the calcium hydroxide-based dressing. Studies show that EndoREZ may not be as biocompatible as others sealers [27-29]. *In vivo* biocompatibility of EndoREZ, when applied into the root canals, has not been investigated, but *in vitro*, our results are in agreement with previous

research that showed that EndoREZ sealer is considered as moderate cytotoxicity when in contact with L929 mouse fibroblasts [29].

Our results emphasize the importance of the calcium hydroxide root canal dressing in the root canal treatment of teeth with necrosis and periapical periodontitis [30]. In these groups, there was a decrease in the number of inflammatory cells after 90 days, reduction of areas of bone and root resorption and presence of partial apical sealing in approximately 75% of cases. Considering the importance of the calcium hydroxide intracanal dressing, it was used in all experimental groups. However, despite that, the histological results did not show a reduction of inflammatory infiltrate or bone loss following root canal treatment in teeth with apical periodontitis.

For histological evaluation, we used the periods recommended by ISO 7405:2018 [20]. Although this study was carried out in the 90 day experimental period, the results of our study corroborate whit previous histopathological studies that evaluated endodontic sealers for longer periods. Tanomaru Filho et al. [31] evaluated the repair process in induced periradicular periodontitis in the teeth of dogs root filled with a calcium hydroxide (Sealapex) or a zinc oxide-eugenol (Fill Canal) root canal sealer and reported that there was no complete sealing of the apical opening with mineralized tissue after 180 days. Moreover, they reported that after 270 days, histopathological analysis showed better apical and periapical repair in the teeth filled with Sealapex [31].

In this study, we evaluated the cytotoxicity of Sealapex and EndoREZ and their ability to activate J774.1 macrophages primed or not with LPS as measured by expression of TNF-α pro-inflammatory cytokine. Sealapex presented low cytotoxicity at 24 hours, while EndoREZ presented toxicity higher than recommended by ISO (maximum of 30% of cell death) [20]. Previously it has been shown that Sealapex did not stimulate peritoneal macrophage cells or inhibit J774.1 macrophages to release TNF-α [22,32]. The impact of that could be detected *in vivo*, because Sealapex treatment showed an improved regulation of inflammatory reaction apically detected by means of bone loss measurement and inflammatory cell recruitment. *In vivo* studies have demonstrated that Sealapex root canal sealer showed satisfactory biocompatibility when implanted in subcutaneous tissue of mice [33] and when used for root canal filling in dogs teeth where they induced a complete apical sealing with deposition of mineralized tissue [14].

Conclusion

Histopathological findings indicate that Sealapex allowed improved tissue repair following root canal treatment in teeth with apical periodontitis compared to EndoREZ. However, a delayed repair could result in the increased toxicity of EndoREZ and induction of TNF-α gene expression by macrophages.

Authors' Contributions

LABS	https://orcid.org/0000-0001-7118-6859	Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review and Editing and Visualization.
KCB	---	Conceptualization, Methodology, Formal Analysis and Investigation.
FSL	https://orcid.org/0000-0001-9995-5613	Writing - Review and Editing.
GGM	https://orcid.org/0000-0001-7021-0028	Writing - Review and Editing.
DSHF	https://orcid.org/0000-0003-4563-4958	Methodology, Investigation, Data Curation and Writing - Review and Editing.
MRL	https://orcid.org/0000-0003-2802-9983	Methodology, Investigation and Writing - Original Draft.
RABS	https://orcid.org/0000-0002-0230-1347	Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review and Editing and Supervision.
FWGS	https://orcid.org/0000-0001-8559-532X	Methodology, Writing - Review and Editing and Supervision.

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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