Evaluation of tissue reaction to freshly mixed endodontic sealers and their relationship with the M1 macrophage phenotype

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ABSTRACT

Introduction: This study assessed tissue response to freshly mixed sealers (Sealer 26 – SE26 and Epiphany - EPH) and the relationship between M1 macrophages and tissue repair. **Methods:** A total of 21 rats were used to assess tissue response at 7, 14 and 21 days post-implantation. Half of histopathological sections were labeled with anti-iNOS antibodies (M1 macrophage subset) and half were graded according to the inflammatory intensity. **Results:** On day 7, no significant difference was found in the intensity of the inflammatory reaction between sealers. On days 14 and 21, SE26 showed a more intense inflammatory reaction than EPH ($P < 0.05$). On days 7 and 14, the number of iNOS+ cells was statistically higher for EPH than SE26 ($P < 0.05$). **Conclusions:** SE26 was more toxic than EPH. The degree of inflammation observed in EPH had an inverse relationship with the amount of M1 macrophages observed.

Keywords: Biocompatibility. Endodontic sealers. Macrophages. Tissue reaction.

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Introduction

Endodontic sealers can inadvertently overflow beyond the apical foramen into surrounding soft and hard tissues,[1,2] causing severe inflammation. Taken together with irritation caused by the pathological process itself, inflammation may lead to tissue necrosis in this area, which compromises endodontic treatment success.[3] Furthermore, sealers or degradation products may gain access to periodontal tissue[4] and participate in the development of periapical inflammation.[5] Thus, biocompatibility of a specific root canal sealer remains one of the major considerations when selecting an appropriate sealer for endodontic use.[6]

Several methods have been used to assess the biocompatibility of endodontic sealers,[7,8,9] including histopathological examination of connective tissue response around the implanted material.[8] Inflammatory response is determined by the presence of neutrophils, lymphocytes, macrophages and mast cells.[8,10] Macrophages are the predominant cells present in inflammatory infiltrates released in response to endodontic sealers.[10]

Over the last decade, several researchers have hypothesized that macrophages develop into two major functional subsets that display inflammatory *versus* anti-inflammatory patterns.[11,12] Accordingly, the presence of a certain subtype of macrophage in the connective tissue after injury could lead to either faster repair or persistence of inflammatory reactions for a longer period of time.[13] These macrophages, known as M1 and M2, can both be found in tissue, but the predominance of M1 is generally associated with greater inflammatory reaction and release of pro-inflammatory mediators,[14] while M2 is associated with the initiation of tissue remodeling, angiogenesis and repair.[15]

Despite the importance of biomaterial compounds in macrophage activity,[16,17] few studies have attempted to assess the effects of endodontic sealers on macrophage phenotype,[10,14,18] which may influence the magnitude, type and duration of the inflammatory immune response. Considering that functional macrophage phenotypes can be altered as a result of changes in their microenvironment,[11,12,13] such as material properties,[10,14,16-18] further studies need to be conducted to determine the effects of chemically different endodontic sealers on referred cells.

Sealer 26 and Epiphany are resin-based endodontic sealers which have been extensively investigated regarding cytotoxicity[19,20] and tissue biocompatibility.[1,3,7,21-24] However, no study has been conducted to analyze the relationship between pro-inflammatory macrophage phenotype and tissue response after subcutaneous implantation of endodontic sealers. Therefore, it is important to assess the potential mechanisms that could result in tissue response to endodontic sealers, for example, material chemical composition that leads to prolonged activation of the macrophage pro-inflammatory phenotypic profile.[17] As such, the aim of this study was to assess the tissue reaction promoted by Sealer 26 and Epiphany in early periods and analyze the presence of M1 macrophages according to the type of sealer, as well as the relationship between the M1 macrophage profile and tissue repair.

Material and methods

For the experiments, 21 male Wistar rats (*Rattus norvegicus*) were used and weighed between 300 and 400 g at the time of surgery. The animals were divided into three groups of seven animals and anesthetized by intraperitoneal administration of 50mg/kg ketamine HCl (Cetamin®- Rhobifarm, SP, Brazil) and 7mg/kg xylazine (Anasedan®, SP, Brazil). The backs of the animals were shaved and disinfected with 0.5% chlorhexidine gluconate. All protocols were performed in accordance with the Research Ethics Committee of the University of Uberlândia (CEUA-Protocol #016/09).

Two separate pockets were created by blunt dissection to a depth of 20 mm in order to implant the material in the subcutaneous tissue. Sterilized polyethylene tubes (Embramed, SP, Brazil), 10-mm long with an inner diameter of 1.5 mm, were filled with freshly mixed resin-based sealers (Sealer 26® - SE26, Dentsply Ind. Ltda, Petrópolis, Brazil and Epiphany® - EPH, Pentron Clinical Technologies, Wallingford, USA), prepared according to the manufacturer's recommendation and then placed into each pocket. The surgical wounds were closed with ethyl cyanoacrylate (Super Bonder, Loctite, Itapevi, Brazil). All procedures were performed under strict aseptic conditions.

After 7, 14 and 21 days, the animals were killed by an overdose of thiopental sodium (150 mg/kg).

The material implanted in the surrounding tissues was removed, immersed in 10% neutral buffered formalin for 48 hours and embedded in paraffin. The connective tissue adjacent to the open end of each tube was subjected to semi-serial 5-µmin longitudinal sections which passed through the opening of the polyethylene tube and the interface between the material and the connective tissue. The sections were stained with Hematoxylin and Eosin (HE) for histopathological analysis and subjected to immunohistochemical analysis for M1 identification. The connective tissue alongside the lateral wall outside the polyethylene tubes was used as control.

Immunohistochemistry was performed on five sections of each specimen to assess the M1 macrophage phenotype that surrounded the implant/connective tissue interface. To this end, following deparaffinization, the samples were treated with 0.25% trypsin (Sigma-Aldrich Co., St Louis, USA) for 30 minutes, placed in a solution of 3% H_2O_2 in methanol for 50 minutes at room temperature, and incubated with Rodent Block R (Biocare Medical, Concord, USA). Subsequently, the sections were incubated with a mixture of XR Factor (Biocare) and primary antibody to M1 phenotype (anti-iNOS) at 1:80 dilution (Neomarkers, Fremont, CA, USA) for one hour. Immunoreaction was made visible using a labeled streptavidin-biotin kit (HRP Rabbit on Rodent, Biocare) for 30 minutes, and positive reactions were detected with diaminobenzidine chromogen (DAKO, Carpenter, California, USA). The sections were counterstained with hematoxylin. As a positive control, primary antibody with PBS was analyzed as described above. Negative staining indicated the specificity of the eliminated antibody.

Histomorphometric analysis

Histopathological analyses were performed under a light microscope (Carl Zeiss, Oberkachen, Germany) at 400× magnification on the basis of the tissue responses stimulated by the tested material and the lateral wall of the tubes (control group). Evaluation of the inflammatory reaction was carried out in three different areas of each HE section. The following was assessed: presence or absence of inflammatory infiltrate (polymorphonuclear cells and mononuclear cells), macrophage activity (macrophage and giant inflammatory cells), mast cells, dispersed material and necrotic tissue. The scores used to quantify the presence or absence of these events were as follows: (–) absent, (+) slight, (++) moderate, and (+++) intense. Depending on these features, a grade from 1 to 4 was given to rate the inflammatory reaction:

1 = Absent: no chronic inflammatory cells;

2 = Slight: few inflammatory cells scattered in the connective tissue;

3 = Moderate: a large number of inflammatory cells focally arranged ;

4 = Severe: a large number of inflammatory cells diffused in connective tissue.

Quantitative immunohistochemical analyses in each experimental period included the assessment of the total number of iNOS+ cells (M1 macrophages) per visual field for each sealer. Three fields were randomly chosen per section and images were digitized under 400x magnification using a Nikon Eclipse E200 microscope (Nikon Instruments Inc., New York, USA) connected to an Evolution MP Color Camera (Media Cybernetics Inc. Bethesda, USA) using Image-Pro Plus 7.0 software (Media Cybernetics). The amount of immunopositive cells (cytoplasm stained with anti-iNOS primary antibody) was analyzed at the tube/connective tissue interface. Staining of the extracellular matrix was not assessed.

The inflammatory reaction and M1 staining results were assessed by means of Mann–Whitney U and Kruskal–Wallis nonparametric tests. Significance was set at P < 0.05.

Results
Analysis of inflammatory reaction
Qualitative analysis
7-day results

SE26. Most specimens presented with a large amount of dispersed material in the connective tissue and a high degree of erythrocytes overflow. Severe inflammatory reaction was predominantly formed by scattered mononuclear cells and active microcirculation with blood stasis (Fig 1A).

Additionally, the presence of some small foci of tissue degeneration, rare macrophages and polymorphonuclear cells, and the absence of giant cells and presence of small groups of mast cells near to blood

vessels were also found. At this point, the formation of an organized fibrous collagen capsule was not found.

EPH. Severe inflammatory reaction was also observed in this group. It was predominantly formed by mononuclear cells. In general, the same characteristics mentioned for Sealer 26 were also identified, except for the greater amount of mast cells and increased organization of connective tissue around the material, which indicates the beginning of fibrous capsule formation (Fig. 1B). The control in both groups showed mild inflammation with a marked presence of tissue organization near the sidewall of the tube.

14-day results

SE26. The intensity of inflammation ranged from moderate to severe (Fig 2A). The presence of mononuclear cells, mast cells, macrophages and giant

cells (only in one specimen) were noted. Capsule formation with the presence of cells, blood vessels and collagen fibers was also found. The presence of necrosis was restricted to areas of direct contact with the sealer.

EPH. Moderate to slight inflammatory reaction, with mononuclear cells, macrophages and giant foreign body cells was present. Some areas were populated by mast cells and hyperemic dilated blood vessels (Fig 2B). The extracellular matrix was more organized than in the Sealer 26 group. For both sealers, the control showed no inflammation, and was characterized by fibrous tissue.

21-day results

SE26. The absence of mild or moderate inflammation was observed. Macrophages appeared in regions where there was greater contact between
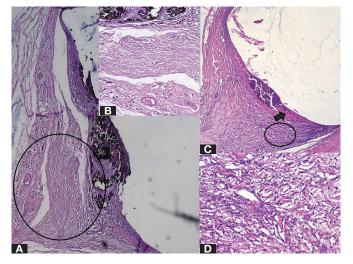

Figure 1. Histological aspects observed in SE26 (**A**) and EPH (**B, C**) at 7 days. **A**) SE26 presents material dispersed in the connective tissue and increased foci of tissue degeneration (10X). Area evidenced by circle is showed on right (20x). Note the presence of vessels, some mast cells and necrotic tissue near the sealer. **B**) EPH shows the beginning of capsule formation (4X, arrow). **C**) High magnification of EPH (40X) evinces scattered mononuclear cells and active microcirculation with blood stasis.
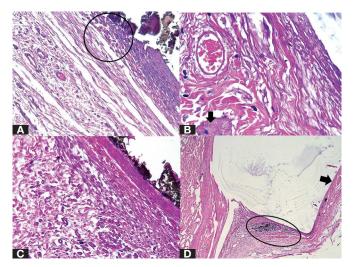

Figure 2. Histological aspects of SE26 and EPH at 14 (**A, B**) and 21 (**C, D**) days, respectively. **A**) SE26 presents mononuclear cells and blood vessels. Capsule formation (arrow, 20X). Circle evinces direct contact between sealer and tissue. **B**) EPH shows areas populated by mononuclear cells, macrophages and giant foreign body cells (black arrow 20X). Capsule is completely formed. **C**) SE26 presents mononuclear cells near the contact between connective tissue and sealer (20X). Fibrous capsule with cells. **D**) EPH shows an organized fibrous capsule (elliptical contour) around the remnant sealer, collagen fibers (4X). Arrow shows the control, which corresponds to the lateral teflon wall.
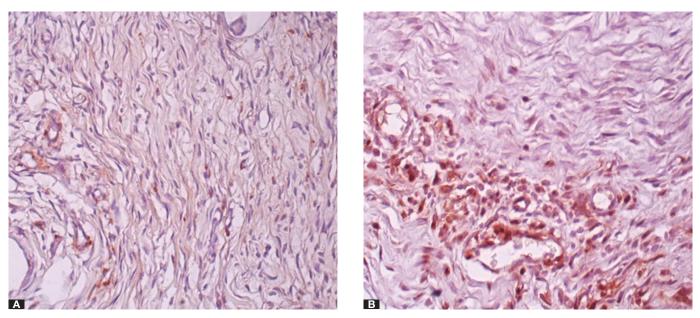
Figure 3. Representative images of cells stained with anti-iNOS antibody (**A** = SE26 group; **B** = EPH group). Note that endothelial cells also stain with anti-iNOS (**B**) Not included in cell counting.

connective tissue and material overflow. Foreign body giant cells and macrophage-engulfed sealer were present (Fig 2C). Though slender in most specimens, the fibrous capsule was complete, with blood vessels present.

EPH. During this period, there was mild to absent chronic inflammatory reaction. Macrophages were observed in areas of residual dispersed material. The establishment of a fibrous capsule was evident (Fig 2D).

Quantitative analysis

On day 7, there was no significant difference on the intensity of the inflammatory reaction between the SE26 and EPH (P > 0.05). However, on days 14 and 21, there was significant difference between groups, with SE26 showing the worst response (14 days, P = 0.0042; 21 days, P = 0.00109). When analyzed over time, SE26 showed statistically significant reduction in inflammatory reaction from the 14th day on (P = 0.002), whereas EPH showed a significant reduction in the intensity of inflammation from the 7th day on (P = 0.0003).

Analysis of M1 stained cells

Results indicated that M1 macrophages (iNOS+ cells) were present in both sealers in all study periods. However, the number of iNOS+ cells was statistically higher in the EPH group on days 7 and 14 (7 days, P = 0.0079; 14 days, P = 0.0115). On day 21, no statistically significant differences were detected between sealers (P = 0.6555). In the SE26 group, there were no statistically significant differences on the number of iNOS+ cells (P = 0.3126) over time. In the EPH group, the number of iNOS+ cells varied depending on the time, and showed significant differences between 7 and 14 days (P = 0.0246). The EPH group showed a higher number of iNOS+ cells per field at 14 days. Figure 2 shows representative images of iNOS+ cells in SE26 and EPH groups.

Discussion

The present study examined the effects of the chemical composition of two freshly resin-based endodontic sealers on the inflammatory response following implantation, as well as the relationship between the presence of pro-inflammatory macrophage

subset (M1) and the host's tissue response until 21 days post-implantation. Although several experimental studies have assessed the biocompatibility of Sealer 26 and Epiphany, divergent reports are found in the literature.[1,18] Some authors have demonstrated sealers good biological behavior,[1,2,23] whereas others have shown severe tissue reaction.[3,22,24] According to Scarparo et al,[25] these contradictions apparently arise from the many different experimental designs and methods used to assess inflammatory reactions. In the present study, the high toxicity presented by SE26 on days 14 and 21, when compared to EPH, is likely related to differences in material composition because SE26 is an epoxy resin-based sealer containing calcium hydroxide.[1]

Although both sealers contain calcium ions (Ca2+), the amount of ion is greater in SE26, which may potentiate the cytotoxic effect of the resin compound in the initial hours before complete setting. Ca2+ ions released by endodontic sealers that contain calcium hydroxide are implicated in many cellular functions, inducing the activation of cellular cascades related to cytokine and growth factors released by neutrophils and macrophages.[3] Appropriate tissue response depends on the nature and amount of this ion, which might accelerate the repair processes and reduce inflammatory responses in periapical tissue[26] or result in cell apoptosis and tissue necrosis. Whereas neither the amount of Ca2+ ions released by the sealers studied before setting nor the levels of Ca2+ that determine a favorable or unfavorable healing are known, several questions about the mechanisms that lead to differences in the biological response to the two sealers remain open. Additionally, it is important to emphasize that the areas of late repair in SE26 at 21 days corresponded to regions featuring overflow of sealers, which is in agreement with the findings of other authors.[2,23]

With regard to M1 macrophage labeling (iNOS+ cells), a higher number of iNOS+ cells was expected in the group with greater intensity of inflammatory reaction. Conversely, EPH presented the highest number of positive cells, which may be related to the role of these cells in the release of enzymes related to matrix degradation, such as collagenases and metalloproteinases[12,15] and their influx into regions inflamed by debris removal.[11,12] Therefore, a greater number of iNOS+

cells in the EPH group could allow faster remodeling and removal of sealer debris, which could be related to faster tissue reorganization observed in sections stained with HE. Nevertheless, it is important emphasize that the differentiation between M1 and M2 profiles represents two extremes within continuous functions performed by macrophages.[11,12,13] In other words, the greater number of M1 macrophages in the EPH group does not necessarily mean that they are producing the same mediators, as the M1 macrophages, in the SE26 group because the behavior of these cells depends on the antigens or substrates present.[16] As such, we speculate that the excess debris and necrotic tissue in the SE26 group could have prevented those cells from the cleaning process, which prolonged the activation of macrophages, in turn, releasing pro-inflammatory mediators and delaying repair.

When assessed over time, EPH presented a greater amount of M1 macrophages on day 14, which reinforces the hypothesis that subtype M1, although essentially pro-inflammatory, is critical to the repair process. On the other hand, SE26 showed no significant differences in the number of M1 macrophages over time, which could indicate that this balance may have hampered repair at the early stages. There are no reports in the literature that support or refute the present findings because this is the first study to use the presence of macrophages with a pro-inflammatory profile as a complementary parameter for assessing the biocompatibility of freshly mixed endodontic sealers.

Despite the results, it is important to address the limitations of the present study. First, M1 staining was based on a unique cell marker (iNOS). Although iNOS is a classic marker for the M1 macrophage, which is not present in M2 cells,[12] and there are many markers that distinguish M1 macrophages in murine and human tissues, most of these markers are not available for analysis of rat tissue, especially formalin-fixed tissue.[16,17] Second, M2 macrophages and intermediate subsets were not labeled. Considering that the selected experimental model aimed to assess only the reaction caused by the sealers in the early stages post-implantation, the study focused on the period in which the sealer components may be responsible for transient toxicity, represented by the labeling of the M1 subtype which is essentially pro-inflammatory.

The outcomes of the present study indicated that SE26 is more toxic than EPH, although the intensity of its inflammatory reaction is likely to decrease with time. The degree of inflammation observed in EPH showed an inverse relationship with the amount of M1 macrophages observed. Further research is necessary to determine the relationship between material composition, tissue inflammation and profile of recruited macrophages.

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