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Incorporating N-acetylcysteine and tricalcium phosphate into epoxy resin-based sealer improved its biocompatibility and adhesiveness to radicular dentine

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ABSTRACT

Objective. This in vitro study was designed to evaluate the biocompatibility, adhesiveness, and antimicrobial activity of epoxy resin-based sealer associated with N-Acetylcysteine (NAC) or beta-tricalcium phosphate nanoparticles (β-TCP) as an experimental retro-filling material.

Methods. Cytotoxicity was assessed using 2,3-Bis-(Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) and Sulforhodamine B (SRB) assays after exposing human periodontal ligament fibroblasts to extracts of the materials for 1, 3, or 7 days. For the adhesive resistance test, root canals (48 single-root teeth) were instrumented with Reciproc #40 files (VDW GmbH, Germany) and obturated. After 7 days, the apices were sectioned and a retrograde cavity prepared and filled with the experimental materials (Mineral trioxide aggregate, Epoxy sealer, Epoxy sealer + NAC, and Epoxy sealer + β-TCP). For the push-out test, one 2-mm thick slice was obtained from the apical third of each specimen. Antimicrobial activity was performed using agar diffusion method. Biofilms were grown in microplates and exposed to the extracts of retro-filled materials, followed by analysis of growth inhibition on agar plates.

Results. Epoxy sealer in association with β-TCP or NAC showed better bond strength while Mineral trioxide aggregate allowed for the lowest adhesion. Mineral trioxide aggregate, Epoxy sealer + β-TCP, and Epoxy sealer + NAC showed low cytotoxicity. Epoxy sealer was the most cytotoxic. In antimicrobial activity assays, all materials had no effect on *Candida albicans*. Addition of NAC improved the antimicrobial property of Epoxy sealer against *Enterococcus faecalis* compared to unmodified Epoxy sealer ($P < 0.05$).

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Significance. Incorporating β-TCP or NAC with Epoxy sealer could improve the adhesiveness and biocompatibility for better use in endodontic therapy.

1. Introduction

Eliminating microorganisms and their by-products from the root canal system is one of the main objectives of endodontic therapy and yet, it has been the greatest challenge [1]. Despite significant progress in developing instrumentation with new techniques and the use of irrigation solutions, mechanical preparation for endodontic therapy is not effective due to the complex and variable anatomy of the root canal. This leads to retention of bacteria and debris in some regions, which are not accessible to the preparation [2].

Poor sealing of the canal system results in the incomplete the constant action of irritants and, thus perpetuates inflammation and infection [3]. In vitro SEM evaluations have shown that microbial penetration in the dentin tubules can reach an average depth of 100 μm, which is difficult to achieve via most endodontic irrigators. For example, while sodium hypochlorite penetrated only 60–150 μm of the dentinal tubules, *Enterococcus faecalis* reached 600–1000 μm [4].

Typically, when conventional root canal treatment fails in clinical situations the preferred subsequent option in most cases is non-surgical retreatment. However, in some instances, other factors, such as a complex root canal system or previous accidents, may interfere with the success of non-surgical retreatment. In such cases, periradicular surgery is the treatment of choice in order to save the tooth [5]. This procedure involves placing the root canal filling material between the infected root canal system and periradicular tissues in order to close the path of communication after performing root-end resection and preparation. Thus, a root canal filling material with ideal properties will allow for better outcomes for this surgical treatment. An ideal root canal filling material is expected to be non-absorbable, non-corrosive, non-cytotoxic, not affected by moisture, dimensionally stable, biocompatible, antibacterial, radiopaque, cost-effective, easily manipulated, able to adhere to the dentinal walls (adhesiveness), create a tight seal, and induce cementogenesis [6].

Epoxy resin-based sealer is the gold standard [7,8] introduced to endodontics by Schroeder [9], has good physical properties, biocompatibility, radiopacity, good adhesion to dentin, and confers long-term dimensional stability [10–12]. However, the properties of Epoxy resin-based sealer could be further improved by adding additional components and/or compounds.

First is *N*-acetylcysteine (NAC), which is an antioxidant and a precursor to glutathione that modulates intracellular signaling during inflammation, and decreases its own synthesis and the release of pro-inflammatory molecules, including cytokines and prostaglandin E2 [13]. Second is beta tricalcium phosphate (β-TCP), which is resorbable and provides a conducive environment for the growth and maintenance of new bone tissue, which facilitates bone mineralization [14]. Thus, NAC or β-TCP could potentially improve the biological and physical performance of an Epoxy sealer, while not necessarily changing its physico-chemical or biological properties. Therefore, the aim of this in vitro study was to evaluate the biocompatibility, adhesiveness and antimicrobial activity of a retro-filling material consisting of Epoxy resin-based sealer and NAC or β-TCP.

2. Material and methods

2.1. *Retro-filling materials*

Epoxy resin-based sealer (AH Plus - Dentsply Maillefer, Tulsa, OK, USA), Mineral trioxide aggregate (MTA - Angelus Industria de Produtos Odontologicos, Londrina, PR, Brazil), NAC (N-acetylcysteine, Sigma–Aldrich, São Paulo, SP, Brazil) and β-TCP nanocrystals (particle size 20–550 nm, Berkeley Advanced Biomaterials, Inc., Berkeley, CA, USA) were used in this study. The retro-filling materials were prepared according to the manufacturers' instructions. Epoxy resin-based sealer was associated with NAC or β-TCP at a ratio of 1:4, forming the following groups: Epoxy sealer + NAC and Epoxy sealer + β-TCP. Epoxy sealer (unmodified) and Mineral trioxide aggregate were also tested for comparison purposes.

2.2. *Push-out test*

Forty-eight intact human teeth, extracted for orthodontic or periodontal reasons, were used in this test. The teeth were cleaned, placed in saline solution (details of solution such as pH), and frozen at −4 °C. The crown of each tooth was sectioned at the cement-enamel junction using carburundum discs (KG Sorensen, Barueri, SP, Brazil) while cooling with water. Then, all roots were cut 2 mm from the apex [15]. Working length was obtained by visually subtracting 1 mm from the length of a Kerr file #10 (Dentsply Maillefer, Ballaigues, Switzerland) of the main foramen. Root canals were instrumented using the RECIPROC #40 file. During biomechanical preparation and with each exchange of file, the canals were irrigated with 5 mL of 2.5% sodium hypochlorite solution. At the end of the preparation, the canals were flushed with 17% ethylenediaminetetraacetic acid (EDTA) for 3 min followed by irrigation with 5 mL of saline solution and were dried using Capillary Tips (Ultradent do Brasil Produtos Odontologicos, Indaiatuba, SP, Brazil) and filter paper cones (DentsplyMaillefer). The teeth were obturated with gutta-percha and Epoxy sealer using lateral condensation [15]. After 7 days, an apicetomy was performed, sectioning 1 mm of the apical third with a diamond disc under water cooling. Then, the root apices were instrumented with ultrasonic tips (Helse Dental Technology), the preparation was performed at a depth of 2 mm and retro-filled with Epoxy sealer, Epoxy sealer + NAC,

Epoxy sealer + β-TCP, or Mineral trioxide aggregate (control). The teeth were maintained in phosphate-buffered saline (PBS, ThermoFisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO_2 for 14 days, which is 3 times longer than the manufacturers' established setting time for each material. The push-out test was then performed in a universal testing machine (EMIC model DL-1000, Equipamentos e Sistemas Ltda, São José dos Pinhais, PR, Brazil) with a crosshead speed of 1 mm/min and a load cell of 50 Kgf. The adhesive strength (σ) was calculated using the formula: σ = C/A, where C is the load for rupture of test piece (kgf), and A is the interfacial area.

After the push-out test, the fractured specimens were analyzed using a stereomicroscope (Stemi 2000 - Karl Zeiss, Germany) at 50× magnification to determine the nature of the bond failure. Samples were classified according to the following modes of failure: "adhesive failure" between obturation material and dentine, "cohesive failure" within the obturation material, "mixed failure" failure in both the obturation material and dentine. Results of the push-out test were analyzed by ANOVA and statistical significance of the differences between samples was tested by using Tukey and Dunn tests, with a 5% significance level.

2.3. *Cytotoxicity assays*

For cytotoxicity analyses, the various retro-filling materials were first placed in 24-well plates. Then, each well was filled with 2.5 mL of Dulbecco's modified eagle medium (DMEM, Gibco/Life Technologies, Carlsbad, CA, USA) and incubated for 24 h with 5% CO_2 at 37 °C. Thus, extracts of the test specimens were prepared at a ratio of 193 mm^2 sample surface area m^{-1} cell culture medium. After a 24-h extraction period, these original extracts (1:1) were then serially diluted in cell culture medium prior to testing.

2.3.1. *XTT assay*

Human periodontal ligament fibroblasts (CC-7049, Lonza, Walkersville, MD, USA) were cultured in DMEM (Gibco/Life Technologies), supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Lonza) at 37 °C with 5% CO_2.

Cells (8×10^3/well) were seeded in 96-well plates (Corning®, Sigma–Aldrich, Brazil) and incubated for 24 h at 37 °C with 5% CO_2. Then, 200 μL of original extracts (1:1) and serial dilutions (1:2, 1:4, and 1:8) were added to the cell cultures. After 24 h, cell viability was determined by the XTT (2,3-Bis-(Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide) assay. Briefly, the conditioned media in the wells were replaced with 200 μL of the XTT solution (ThermoFisher Scientific, Waltham, MA, USA) and the plates were incubated for 3 h at 37 °C with 5% CO_2. Then, optical density (OD) was measured at 490 nm in a plate reader (Asys-Hitech-GmbH, Eugendorf, Austria). Three independent experiments were carried out with four replicates of the cell cultures used for each treatment. OD values were normalized to untreated control cultures (=100%) and differences between median values were statistically analyzed using ANOVA, Tukey, and Dunn tests (Graph Prism 6.0, GraphPad Software Inc., La Jolla, CA, USA) at a 5% significance level.

2.3.2. *SRB assay*

Human periodontal ligament fibroblasts (CC-7049, Lonza) were cultured in DMEM (Gibco/Life Technologies), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Cells were seeded in 96-well plates at a density of 8×10^3/well and incubated for 24 h at 37 °C (5% CO_2). Then, 200 μL of original extracts (1:1) and their serial dilutions (1:2, 1:4, and 1:8) were added to the cell cultures. After 24 h, the conditioned media were removed, and cells were washed with 100 μL PBS. Then, 100 μL of 10% trichloroacetic acid (Sigma–Aldrich) was added to each well and the plates were refrigerated at 4 °C for 1 h. The wells were then washed and after complete drying, 100 μL of 0.4% Sulforhodamine B (SRB, Sigma-Aldrich) was pipetted into each well. After 1 h at room temperature, the plates were washed with 1% acetic acid (Sigma–Aldrich), and 100 μL of tris base (Trizma® Sigma–Aldrich) was added per well. Finally, OD values were measured at 570 nm using a plate reader (Asys Hitech-GmbH). Three independent experiments were carried out with four replicates of the cell cultures used for each treatment. OD values were normalized to untreated control cultures (=100%), and differences between median values were analyzed using ANOVA, Tukey, and Dunn tests at a 5% significance level.

2.4. *Antimicrobial analysis — agar diffusion test*

Reference strains of *Candida albicans* (ATCC 18804), *E. faecalis* (ATCC 4083), and *Escherichia coli* (ATCC 25922) were obtained from the Institute of Science and Technology/UNESP.

The various retro-filling materials were placed in 24-well plates and each well was filled with 2.5 mL sterile PBS. After incubation for 24 h with 5% CO_2 at 37 °C, filter paper discs (5 mm in diameter) were soaked in the original extracts and left to dry in an incubator at 37 °C.

The discs were then placed in petri dishes in direct contact with *E. faecalis* in Brain Heart Infusion broth (BHI) agar (Himedia, Mumbai, India), *E. coli* in *MacConkey* agar (Himedia), and *C. albicans* in Sabouraud dextrose agar (SD– Himedia) for 24 h at 37 °C.

Two discs were placed per dish and four independent experiments were performed. Control discs were placed in contact with 2% chlorhexidine gel (Bioformula, São José dos Campos, SP, Brazil). Diameters of the microbe free area in each filter paper disc were then measured and the values were analyzed by ANOVA and statistical significance of the differences between samples were analyzed by Tukey and Dunn tests, at a 5% significance level.

3. Results

3.1. *Push-out test*

Results of the push-out test showed that the adhesive strength of Epoxy sealer + NAC and Epoxy sealer + β-TCP was significantly higher to the root canal walls compared to Mineral trioxide aggregate and the control group (P = 0.0001). Moreover, Epoxy sealer + β-TCP demonstrated an increase in bond

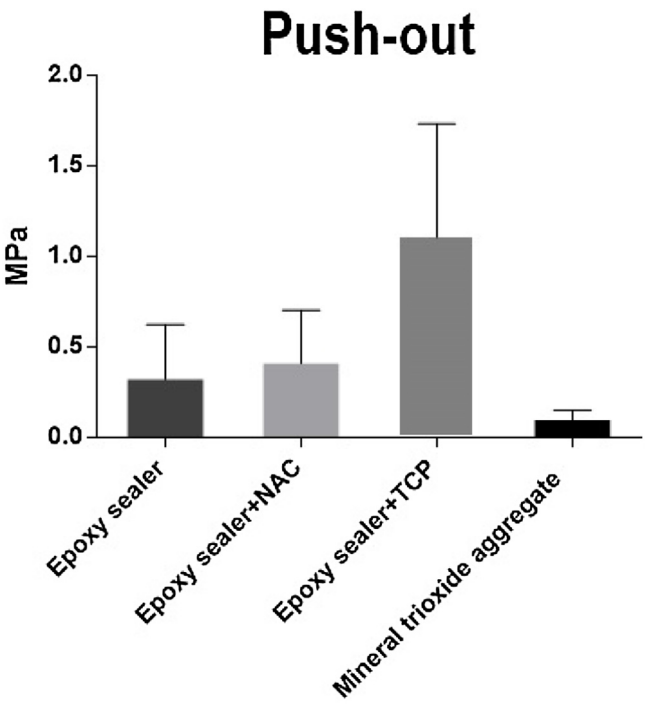

Fig. 1 – Mean (+SD) of push-out bond strength of various sealers at coronal, middle and apical thirds of root canal in MPa.

strength compared to unmodified Epoxy sealer (P = 0.0001) (Fig. 1).

It was observed by stereomicroscope that Epoxy sealer presented predominantly dentin cohesive failure. Most failures for Epoxy sealer + β-TCP and Epoxy + NAC were mixed (dentin and cement), while more adhesive failures were noted for Mineral trioxide aggregate.

3.2. *XTT assay*

In the XTT test, Mineral trioxide aggregate was found to be the least cytotoxic material, among the groups (P = 0.001) promoting more than 90% cell viability, and higher cellular proliferation. In contrast, significantly lower cell survival rates were found in 1:1 and 1:4 dilutions of Epoxy sealer on day 1, 3 or 7 compared to the other retro-filling materials (P < 0.01) (Fig. 2), indicating that Epoxy sealer was the most cytotoxic material. Moreover, Epoxy sealer + NAC and Epoxy sealer + β-TCP promoted cell viability at similar levels and exhibited high cell proliferation at all dilutions and in all three time periods tested (P > 0.05).

3.3. *SRB assay*

In the SRB test, Epoxy sealer + β-TCP and Mineral trioxide aggregate were found to have the lowest cytotoxic effects, showing more than 90% cell viability and higher cell proliferation. Epoxy sealer + NAC showed lower cell proliferation than Epoxy sealer + β-TCP and Mineral trioxide aggregate in all dilutions from 1:1 to 1:4 dilutions on day 1 (P < 0.01). However, Epoxy sealer presented the lowest cell viability from 1:1 to 1:4 dilutions compared to all other materials tested (P < 0.01) (Fig. 3).

3.4. *Agar diffusion*

Antimicrobial activity of the retro-filling materials was evaluated by the microbial growth inhibition assay. Fig. 4 shows that *E. coli* growth was highly inhibited by Mineral trioxide aggregate (P = 0.001) followed by Epoxy sealer + β-TCP and Epoxy sealer. Moreover, higher inhibition of *E. faecalis* growth was achieved by Mineral trioxide aggregate and Epoxy sealer + NAC than by Epoxy sealer + β-TCP and Epoxy sealer (P = 0.001). Interestingly, none of the materials tested inhibited the growth of *C. albicans*.

4. Discussion

Adhesiveness is an important characteristic of endodontic sealers since it results in a tight seal [16]. Although a number of studies [7,11,14,16] have evaluated the adhesiveness of sealers, only a few have investigated [8,17] them as retro-filling materials.

In this study, we found that the adhesiveness of Mineral trioxide aggregate was inferior when compared to the other materials evaluated. This is in agreement with the findings of Silva et al. [16] who showed that Mineral trioxide aggregate presented a lower seal quality compared to Epoxy sealer because it could infiltrate and thus reduce material adhesion. In contrast, Oliveira et al. [18] MTA-filled specimens had higher push-out bond strength than AH Plus, iRoot SP, and MTA Fillapex. Also, Assmann et al. [19] found no difference between Epoxy sealer and Mineral trioxide aggregate with respect to bond strength with dentin when analyzed by the push-out test.

The difference observed in the present study between Mineral trioxide aggregate and the other materials could be likely due to the use of materials without gutta-percha and also because only the apical part was evaluated. Notably, our study found that the association of β-TCP or NAC to Epoxy sealer increased adhesiveness of the materials to the root canal walls.

Among the materials tested, Epoxy sealer was found to be the most cytotoxic based on the results of the XTT and SRB assays, regardless of the tested time (1, 3 and 7 days). It should be noted that cytotoxicity decreased in high dilutions. On the other hand, Mineral trioxide aggregate increased cell proliferation in all dilutions and at all times tested. These results are consistent with the study by Bin et al. [20] who showed that Mineral trioxide aggregate was the least cytotoxic material, promoting higher cell viability in the XTT test. Also, Epoxy sealer presented intermediate cytotoxicity at high dilutions, but cell survival rates in low dilutions were drastically reduced. In contrast, Teixeira et al. [21] evaluated the cell viability of Epoxy sealer and Mineral trioxide aggregate using the MTT assay and showed that Epoxy sealer presented higher cell viability than Mineral trioxide aggregate after 48 h.

Our data revealed that Epoxy sealer + β-TCP supported high cell proliferation at all time periods and dilutions based on the SRB assay data. The association of β-TCP to Epoxy sealer

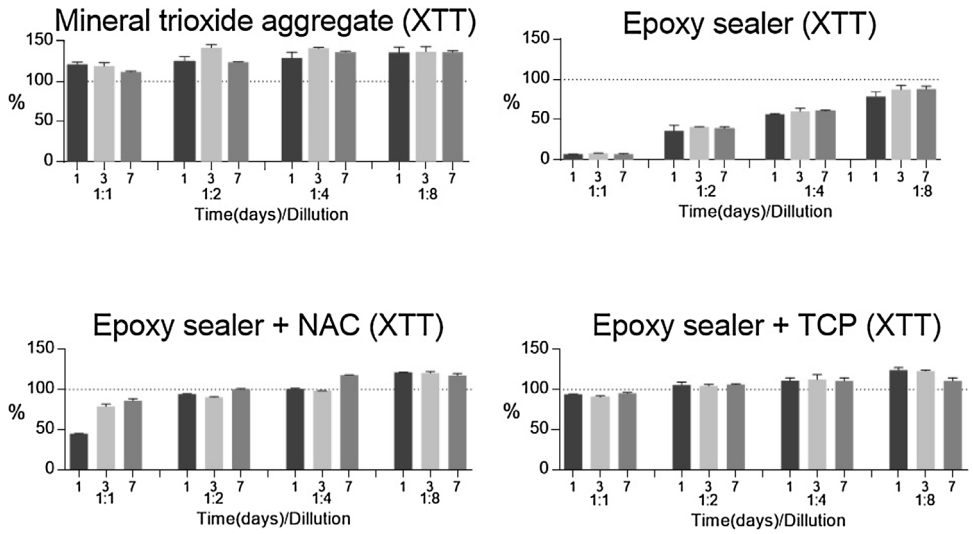

Fig. 2 – Determination of the mean rate of cell viability by XTT test of each group after 1, 3 and 7 days. Data expressed as mean ± mean standard error.

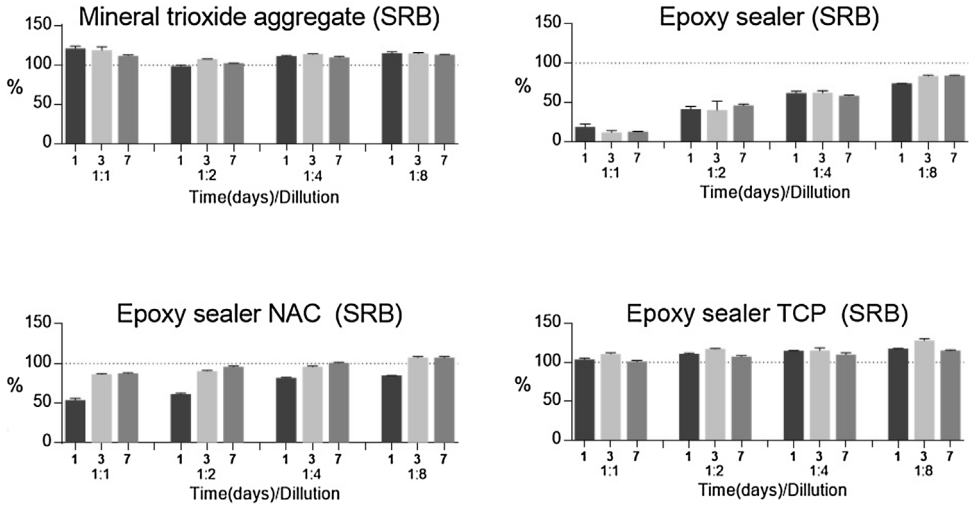

Fig. 3 – Determination of the mean rate of cell viability by SRB test of each group after 1, 3 and 7 days. Data expressed as mean ± mean standard error.

supported greater cell proliferation compared to unmodified Epoxy sealer. This finding could be likely because β-TCP is known to induce bone tissue formation [14]. Portella et al. [22] evaluated an experimental endodontic sealer based on glycerol resin salicylate and α-phosphate tricalcium which induced cell proliferation after 48 h similar to those induced by Epoxy sealer, which is the gold standard.

In the present investigation, Epoxy sealer + NAC showed higher cell viability compared to unmodified Epoxy sealer based on the XTT and SRB tests. The cytotoxicity of Epoxy sealer + NAC decreased from low to high dilutions, presenting the best results on day 7. These results are likely due to the properties of NAC that may have increased the cell viability of periodontal ligament cells. Thus, our results showed that the association of Epoxy sealer with β-TCP or NAC was not detrimental to the periodontal ligament cells, suggesting that Epoxy sealer with β-TCP or NAC could be used as biologically safe retro-filling materials in endodontic therapy.

Finally, the present study demonstrated that Epoxy sealer could effectively inhibit the growth of *E. coli*, and its association with β-TCP demonstrated similar antimicrobial activity. On the other hand, Epoxy sealer + NAC showed decreased antimicrobial activity against *E. coli*. In a previous study [23], also demonstrated that Epoxy sealer showed high antimicrobial activity against *E. coli*. Moreover, Mineral trioxide aggregate presented higher antimicrobial activity against *E. coli* and *E. faecalis* compared to Epoxy sealer and Epoxy sealer + β-TCP. However, the antimicrobial activity of Epoxy sealer + NAC against *E. faecalis* was similar to Mineral trioxide aggregate. Furthermore, none of the materials tested displayed antimicrobial activity against *C. albicans*. In contrast, Miyagak et al. [24] found that Mineral trioxide aggregate and Epoxy sealer

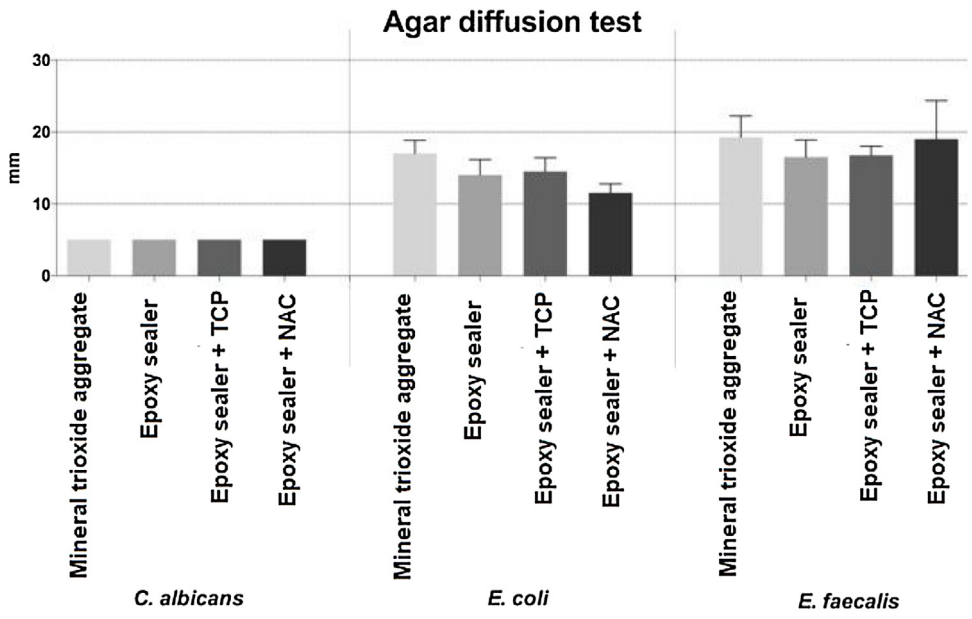

Fig. 4 – Antibacterial activity of each group evaluated by agar diffusion test. All the assays were conducted in triplicate and the results were recorded in terms of the average diameter of inhibition zone (mm). Error bars indicate standard errors of the means.

partially inhibited *C. albicans* and *E. faecalis*, although they could not inhibit their growth completely. Also, CHX and α-TCP addition at an experimental methacrylate-based root canal sealer influenced the physicochemical properties and provided antibacterial properties against *E. faecalis* [25].

The findings reported in this study indicate that β-TCP or NAC can be used along with Epoxy sealer to achieve improved adhesiveness and biocompatibility. Thus, using a resin epoxy-based sealer together with β-TCP or NAC may be an effective approach to achieve a hermetic seal and stimulate healing of periapical tissues. Additional studies are warranted to further optimize the physico-chemical and biological properties of these materials, since they will remain in contact with apical tissues for long periods.

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