

Evaluation of Cell Culture Cytotoxicity of 5 Root Canal Sealers: Release of Hydrogen Peroxide

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ABSTRACT

The cytotoxicity of four calcium hydroxide-based root canal sealers (Sealapex, CRCS, Apexit, and Sealer 26) and one zinc oxide and eugenol-based sealer (Fill Canal) was evaluated in rat peritoneal macrophage cultures for the release of hydrogen peroxide. The least cytotoxic sealer was CRCS, followed in increasing order of cytotoxicity by Sealapex, Apexit, Fill Canal, Sealer 26.

UNITERMS

Root canal sealer, cytotoxicity, H₂O₂ release

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RESUMO

A Citotoxicidade de quatro cimentos obturadores à base de hidróxido de cálcio (Sealapex, CRCS, Apexit e Sealer26) e

um cimento à base de óxido de zinco e eugenol (Fill Canal) foi avaliada em cultura de macrófagos retirado de peritônio de rato para liberação de peróxido de hidrogênio. A menor citotoxicidade foi para o cimento CRCS, seguida em ordem crescente de citotoxicidade pelo Sealapex, Apexit, Fill Canal e Sealer 26.

UNITERMOS

Canal radicular, cimentos obturadores; H₂O₂ liberação

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INTRODUCTION

Successful endodontic treatment requires techniques that respect apical and periapical tissues and which is completed with filling of the root canal with inert, dimensionally stable and biocompatible substances^{1,3}. With this in mind, a vast variety of sealing materials have been proposed and used, such as solid cones de gutta percha inserted into the root canal associated with cements or pastes to hermetically seal the root canal². Because these materials are in direct contact with apical and periapical tissues for a prolonged period of time, biocompatibility is of great importance¹. Holland & Souza⁹, 1985, recommend the association of biocompatible sealers based on calcium hydroxide with gutta percha for permanent root canal filling.

The toxicity of commercially available sealers, such as Sealapex, CRCS, Apexit, Sealer 26, and Fill Canal can be analyzed *in vitro* and *in vivo*. *In vitro* experiments present the advantage of the control of factors and experimental variables, that can cause problems during *in vivo* experiments.

In *in vitro* methods in cell cultures, the sealer or its components can cause morphological changes that can be analyzed with a light, electronic transmission of scanning electron microscope, with or without radioisotope labeling⁴. Toxicity can be determined by intra - and extracellular morphologic changes as well as degenerative processes after cellular exposure to culture material. Morphological changes also indicate the stimulation of the functional ability of protein synthesis, as well as important chemical mediators of the repair process, such as arachidonic acid products, cellular growth factors, cytokines and other substances such as H₂O₂ and NO which can represent a degenerative process or activity. The greater the release of these substances the more cytotoxic is the material when in contact with cells^{6, 11, 12}.

Analysis of the activity and the quantity of chemical mediators released in culture assesses these phenomena. The reaction of each specific group of cells can explain the reactions that occur in the apical and periapical regions in an isolated manner leading to more precise analysis. Thus, the objective of this investigation was to evaluate the cytotoxicity of endodontic sealers by determining the release of H₂O₂ using ELISA.

MATERIALS AND METHODS

Macrophages were obtained from two Swiss Albino mice weighing approximately 100g (two months of age). After sacrifice and antisepsis with 0,3% iodoethanol, the peritoneum was exposed using a sterile scissors. Saline solution (0.85% NaCl) was introduced into the peritoneum and after digital massage the solution was removed by aspiration.

This suspension of peritoneal cells and saline was placed in a Neubauer chamber and was centrifuged at 200g for 10min at 200g for 10 min at 4°C to obtain a concentration of 2.4 x 10⁶ cells/ml. After removal of the solution, the cells were resuspended to the original volume in solution containing 140mM NaCl, 10mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.56mM phenol red and type II horseradish peroxidase (0.01 mg/ml).

Four calcium hydroxide-based root canal sealers were tested, Sealapex (Kerr/Sybron, Romulus, MI, USA), CRCS (Hygenic, Akron, OH, USA), Apexit (Vivadent Schann, Liechtenstein), and Sealer 26 (Dentsply, Rio de Janeiro, RJ, Brazil), and one zinc oxide and eugenol-based sealer, Fill Canal (DG/Ligas Odontológicas, Catumbi, SP, Brazil). The solutions of the sealers were obtained by sonic fractionation and pulverization and were then diluted in polyethyleneglycol 400 (18 and 9mg/ml). These aliquots were incubated at 37°C in a humid atmosphere and then 0.1 ml aliquot of each sealer in solution was transferred to 48-well tissue culture plates. Fifty ml of peroxidase was added. Polyethyleneglycol 400 was used as control. The reaction was interrupted after 1h by the addition of 10ml of 4N NaOH. Experiments were done in quadruplicate.

Absorbance was determined with ELISA with a 620-nm filter composed of a solution of phenol red and 4 N NaOH. Results are reported in nanomoles of H₂O₂/2 x 10⁵ peritoneal cells, from a standard curve established in each test, composed of the known molar concentrations of H₂O₂ in buffered phenol red. The non-parametric Kruskal-Wallis test was used to analyze the effect of the factors material and quantity on the variable H₂O₂ release. P < 0,05 was considered statistically significant.

RESULTS

The control group always released less H₂O₂ than the other groups. The least cytotoxic sealer was CRCS, followed in increasing order of cytoto-

xicity by Sealapex, Apexit, Fill Canal, with Sealer 26 always being the most cytotoxic. Sealers with the same classification were statistically similar (Table 1).

Table 1 - Frequency (N), mean point (MP), minimal significant difference (msd) and classification of the sealer

| Material | N | P.M. | Dms | Classification |
|----------------|---|------|------|----------------|
| 9 and 18 mg/ml | | | | |
| CRCS | 8 | 18.2 | 19.9 | A,B |
| Sealapex | 8 | 21.4 | | A,B,C |
| Apexit | 8 | 27.4 | | B,C |
| Fill Canal | 8 | 33.3 | | B,C |
| Sealer 26 | 8 | 42.2 | | C |
| Control | 8 | 4.5 | | A |
| 9 mg/ml | | | | |
| CRCS | 4 | 10.1 | 14.2 | A,B,C |
| Sealapex | 4 | 7.2 | | A,B |
| Apexit | 4 | 15.5 | | A,B,C |
| Fill Canal | 4 | 17.1 | | B,C |
| Sealer 26 | 4 | 22.5 | | C |
| Control | 4 | 2.5 | | A |
| 18 mg/ml | | | | |
| CRCS | 4 | 6.5 | 14.2 | A,B |
| Sealapex | 4 | 11.5 | | A,B |
| Apexit | 4 | 13.5 | | A,B |
| Fill Canal | 4 | 20.5 | | B |
| Sealer 26 | 4 | 20.5 | | B |
| Control | 4 | 2.5 | | A |

Sealers with the same classification were statistically similar. The variables were analyzed statistically with the Kruskal-Wallis test ($p < 0.05$). Additional statistical tests were applied using the minimal significant difference (msd) with which two mean points were statistically equal if the difference between them was less than the mds.

DISCUSSION

Because it is a strong base, calcium hydroxide can denature proteins, releasing hydroxyl ions, making the medium alkaline, causing the hydrolysis of bacterial lipopolysaccharides, competing with bacteria for CO_2 , and principally aiding in the process of mineralization of apical tissues because of release of calcium ions^{9, 15, 18}.

In contact with apical and periapical tissues, calcium hydroxide initially causes necrosis by superficial coagulation of the tissue. From this necrosis, a series of dependent and independent biochemical reactions of calcium hydroxide occur in tissue cells¹⁶. In the remnant pulp of periapical tissue several cellular products are synthesized, especially arachidonic acid, cytokines, and H_2O_2 .

Inflammatory reactions occur after filling because of contact with filling materials or surgical procedures. The first reaction that occurs at the interface of contact is disorganization of free proteins that act on mastocytes and free nerve endings. With the levels of histamine and neuropeptides increased, changes occur in vascular permeability, blood circulation, endothelial adhesion and consequently leukopese⁶.

Due to the large particle size, neutrophils are not able to phagocytose them which also occurs with eosinophils. After the afflux of neutrophils, macrophages begin to migrate, which have slower mobility and migratory capacity due to their size⁵.

Phagocytosis of sealers by macrophages may occur or not, with the sealers being carried to other parts of the organism. Macrophages are not able to phagocytose gutta percha due to its dense structure. In order to phagocytose sealers, macrophages must release cellular products into the medium via regurgitation or oxidative explosion. Among these products are lysozymes, peroxidase, H_2O_2 , and NO which may act on bacterial elements, foreign bodies, tissue components, neighboring cells, or in an autocrine manner. The more aggressive the material the greater the quantity of products synthesized. As an example of this interaction, in the attempt to phagocytose an inert noningestible body, macrophages release cytokines and growth factors that lead to cytodifferentiation of fibroblasts and peripheral collagen synthesis, establishing a fibrous

capsule or mineralized tissue around the area, isolating healthy tissues^{6, 12, 13}.

The macrophage plays an important role in inflammatory repair and immunologic reactions. Its intense capability of synthesis, phagocytosis, and antigenic processing makes macrophages the true orchestrator of cellular reactions. Its elevated presence and easy obtention led us to choose macrophages from the rat peritonium for analysis of the reaction to endodontic sealers after culture. By analyzing the release of H_2O_2 using ELISA, cytotoxicity could be evaluated.

Our results, after solubilization and after only 1h of contact of the sealers with macrophages cultures, showed the most cytotoxic sealer to be Sealer 26, followed in decreasing order of cytotoxicity by Fill Canal, Apexit, Sealapex and CRCS.

Because Sealer 26 is a resin and is not very soluble, it easily retains its elements and is difficult to phagocytose. After solubilization, Sealer 26 releases tetramine hexamethylene which during metabolism transforms into highly toxic formaldehyde¹⁷. Procosol with a composition similar to Fill Canal releases eugenol after solubilization which is highly toxic⁸. Evaluating microscopically this sealer in dog teeth with pulp necrosis and chronic periapical lesion after 270 days, there was no biological sealing in almost 70% of the cases and the interstitial connective tissue in contact with the material presented extensive necrosis¹⁴.

There was a low index of biologic sealing and severe inflammatory infiltrate when Apexit was evaluated after 180 days in dogs with pulp vitality⁸. Sealapex and CRCS, that are more soluble and are made up of smaller particles, are naturally more phagocytosable, which may explain their low toxicity. The chemical components of these sealers also have low toxicity¹⁰.

Our results are in agreement with these results; however, there are no studies reported in the literature that evaluate the cytotoxicity of endodontic sealers in terms of H_2O_2 release. Thus, we were not able to compare our results with pertinent literature. However, under the same solubility conditions, using sonified cements, the release of H_2O_2 is a reliable and valid method for evaluation of cytotoxicity.

REFERENCES

- ARENHOLT-BINDSLEV, D, HORSTED-BINDSLEV, P. A simple model for evaluation of relative toxicity of root filling materials in cultures of human oral fibroblasts. *Endod Dent Traumatol.* v.5, p.219-26, 1989.
- BIGGS J.T., KARNINSKI, E.J., OSETEK EM. Rat macrophage reaction to implanted sealer cements. *J. Endod.*, v.11, p.30-6, 1985.
- BUNTAK-KOBLER, D. et al. Toxicity of calcium hydroxide endodontic sealers implanted in tissue. *Int. Endod. J.*, v.1, p.95, 1994. (Abstract).
- CAMPS, J. et al. Etude de la coherence entre trois modes d'evaluation de la biocompatibilite. *J. Biol. Buccale*, v.20, p.211-7, 1992.
- CATANZARO-GUIMARÃES, S.A., PERCINOTO, C. Effect of some endodontic materials on the influx of macrophages and multinucleated giant cell development in experimental granulomas. *J. Endod.* v.10, p.101-4, 1984.
- COTRAN R.S., KUMAR, V., ROBBINS, S.L. *Robins-patologia estrutural e funcional*. 4 ed. Rio de Janeiro: Guanabara Koogan, 1991.
- FOREMAN, PC, BARNES, IE. A review of calcium hydroxide. *Int. Endod. J.*, v.23, p.283-97, 1990.
- GULATI, N. et al. Cytotoxicity of eugenol in sealer containing zinc-oxide. *Endod. Dent. Traumatol.*; v.7, p.181-5, 1991.
- HOLLAND, R., SOUZA, V. Ability of a new calcium hydroxide root canal filling material to induce hard tissue formation. *J. Endod.*, v.11, p.535-43, 1985.
- LEONARDO R.T. *Avaliação microscópica da reação apical e periapical frente a dois cimentos obturadores de canais radiculares a base de hidróxido de cálcio (CRCS e Sealapex) em dentes de cães*. Bauru, 1992. (Dissertação) Master's thesis. Faculdade de Odontologia de Bauru, Universidade de São Paulo.
- PERTOT, W.J., In vivo comparison of the biocompatibility of two root canal sealers implanted into the mandibular bone of rabbits. *Oral Surg.*, p.73, p.613-20, 1992.
- PICK, E, KEISARI, Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J. Immunol. Methods*, v 38, p.161-70, 1980.
- PICK, E, MIZEL, D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J. Immunol. Methods*, v46, p.211-26, 1981.
- RASQUIN L.C. *Avaliação histopatológica da reparação apical e periapical em dentes de cães portadores de lesão periapical crônica, experimentalmente induzida, após tratamento de canais radiculares e obturação com três cimentos à base de hidróxido de cálcio*. Araraquara, 1997. Dissertação (Mestrado) Faculdade de Odontologia de Araraquara.
- SAFAVI, K.J., NICHOLS, F.C. Effect of calcium hydroxide on bacterial lipopolysaccharide. *J. Endod.*, v.19, p.76-8. 1993.
- SCHRÖDER, U. Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation. *J. Dent. Res.*, v. 64, sp., iss. p.541-8. 1985.
- SILVA, L.A.B. *Cimentos obturadores de canal radicular à base de hidróxido de cálcio. Avaliação histopatológica do reparo apical e periapical em dentes de cães, da resposta inflamatória em tecido subcutâneo de camundongos. Análise do pH, concentração de cálcio total e condutividade*. Ribeirão Preto, 1995. Tese (Livre Docência). Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo.
- TAGGER, M., TAGGER, E., KFIR A. Release of calcium and hydroxyl ions from set endodontic sealers containing calcium hydroxide. *J. Endod.*, v.14, p.878-82, 1988.