Cytotoxicity and sealing properties of four classes of endodontic sealers evaluated by succinic dehydrogenase activity and confocal laser scanning microscopy

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Abstract

The objectives of this study were to evaluate the cytotoxicity and sealing properties of four classes of endodontic sealers (PCS/Kerr, RoekoSeal/Roeko, TopSeal/Dentsply, and EndoREZ/Ultradent). For cytotoxicity testing (MTT method), the materials were either placed immediately in contact with cultured cells or 24 h after setting, then evaluated at three subsequent time points (24 h, 48 h, or 1 wk). For the leakage study, extracted human roots were obturated with acrylic cones and sealers and immersed for 48 h into rhodamine-labeled lipopolysaccharide. The roots were then observed under a confocal laser scanning microscope to estimate (semiquantitatively) the presence of the rhodamine-lipopolysaccharide (LPS) inside the canal. The results showed that cytotoxicity generally increased with time, and that most materials pose significant cytotoxic risks, particularly in the freshly mixed condition. Further, all materials showed significant leakage although there was large variation among teeth. Overall, the silicon-based material (Roeko Seal) was less cytotoxic and more effective in sealing root canals against LPS leakage than [...]
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A complete sealing of the root canal system after cleaning and shaping is critical to successful endodontic therapy (1, 2). The intimate contact between endodontic sealers and adjacent periapical tissues, coupled with inadvertent but common extrusion of sealers into the periapical region during treatment, makes the biological and sealing properties of sealers paramount to clinical success (3).

Several classes of endodontic sealers are currently used in clinical practice, but all have significant limitations. Zinc oxide–eugenol-based endodontic sealers have been used for many years but release potentially cytotoxic concentrations of eugenol (4, 5). Calcium hydroxide-based sealers promote calcification but tend to dissolve over time and compromise the endodontic seal (6, 7). Glass ionomer sealers may bond tooth structure but also may activate the release of prostaglandins in periapical tissues (8). Although resin-based sealers are increasingly gaining popularity, their toxicity and mutagenicity are well documented (9, 10). More recently, silicon-based materials have been developed as root canal sealers, and preliminary clinical data are promising (11, 12).

Microleakage in the root canal system after endodontic therapy has been demonstrated using dye, fluids, bacteria, and radioisotopes as tracers (13, 14). However, a majority of these tracers are different in size and shape from bacteria and endotoxins that cause problems clinically (15, 16). Thus, the relevance of most microleakage studies to clinical problems remains controversial (17). Recent research shows that lipopolysaccharide (LPS) leakage from the root canal system into the periapical tissues is an important consideration of periapical response (18). Because LPS is a major virulence factor of Gram-negative bacterial pathogens and is known to activate inflammatory and humoral immune responses, its penetration inside the root canal should be avoided (19). Teeth with gutta-percha root-end fills have been shown to permit leakage of LPS, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (20). Conventional fluorescence microscopy has shown that rhodamine B-labeled LPS leakage occurs under cemented crowns, but the sensitivity of conventional microscopy for detecting the fluorescent tracer has not been adequate (21).

Microleakage in the root canal system is one hallmark of breakdown of the endodontic sealer, increasing the risk of a cytotoxic response to components of the sealers in periapical tissues. Materials that promote a stable seal of the root canal system over time presumably also carry lower risk of cytotoxicity (22). A variety of methods have
been used to assess cytotoxic response to breakdown products of dental materials, but succinic dehydrogenase (SDH) activity of cells is a well accepted, biochemically based, and commonly used method (23).

Confocal laser scanning microscopy (CLSM) is a non-destructive technique for visualizing subsurface features of tissues, and can be applied to detect fluorescence deep within tissues (24). Since LPS can be labeled with fluorescent dyes and observed using CLSM, this may be useful to detect microleakage of LPS, thereby better measuring the microleakage of endodontic sealers. Using these techniques, the objectives of this study were to evaluate the cytotoxicity and sealing ability of four classes of endodontic sealers because these two properties are critical to their successful clinical use.

**Material and methods**

Four classes of endodontic sealers were evaluated in this study: Pulp Canal Sealer (ZOE-based, lot #0-1300; Kerr Hawe, Bioggio, Switzerland), RSA Roeko Seal Automix (silicone-based, lot #2110841; Roeko, Langenau, Germany), Top Seal (epoxy-based, lot #0111001685; Dentsply De Trey, Konstanz, Germany) and Edo Rez (methacrylate-based, lot #4089; Ultradent Products, South Jordan, UT, USA).

**Cytotoxicity testing**

Materials were prepared according to the manufacturers' instructions under isometric conditions to prevent the risk of biological contamination during the cytotoxicity testing (25). Briefly, the materials were packed into a Teflon mold (1 mm thick x 10 mm diameter, n = 4) and covered on both sides with Mylar sheets (Angst & Pfister, Geneva, Switzerland). Thus, the ratio of the surface area of the discs to the volume of cell culture medium was 1.88 cm² ml⁻¹ as recommended by the International Organization for Standardization (26). Samples were placed in contact with cell monolayers immediately after setting (fresh condition) to provide conditions relevant to those seen by periodontal cells. A second set of test samples was prepared as described above and stored at 37°C for 24 h before being exposed to the cultured cells. This condition (set) would be relevant to the clinical situation observed after setting.

For cytotoxicity testing, the materials were placed in direct contact with Balb/c 3T3 mouse fibroblasts (ATCC CCL 163, clone A31; American Type Culture Collection, Rockville, MD, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal calf serum, 25 mmol L⁻¹ of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, 1 µg ml⁻¹ of gentamicin, 125 units ml⁻¹ of penicillin, 125 µg ml⁻¹ of streptomycin, and 2 mmol L⁻¹ of glutamine (all from Oxoid, Basingstoke, Switzerland). Twenty-four hours prior to the addition of the materials, the cells were plated at a density of 25 000 cells cm⁻² in 24-well plates (Costar, Cambridge, MA, USA) with 1 ml of medium per well. The cells were incubated at 37°C in 5% CO₂/95% air to allow attachment of the fibroblasts to the bottom of the wells.

Cytotoxicity was assessed at several times to determine its trends in the biological response. Cytotoxicity was initially assessed for 24 h. After this interval, the specimens were removed from the cell culture, rinsed twice with sterile phosphate-buffered saline (PBS), then immediately added to a second cell culture (which had been plated with cells 24 h earlier). This second culture was incubated for 24 h and the mitochondrial activity measured after incubation (48-h reading). The specimens were then removed again, rinsed again, and incubated in cell culture medium without cells for 5 d at 37°C and 5% CO₂. Finally, the specimens were rinsed again with PBS and added to a third cell culture that had been plated with cells 24 h before. The mitochondrial activity was then assessed 24 h later (1 wk reading).

At each of the three time points (24 h, 48 h, or 1 wk), the cells were incubated for 24 h in the presence of the materials or Teflon discs (negative control) before assessing cellular activity by measuring SDH activity. The SDH activity, indicative of cellular mitochondrial function, was measured by means of the MTT colorimetric assay (3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide; Sigma, St Louis, MO, USA). Activity was quantified by dissolving the MTT-formazan into 0.1 M NaOH (4.25 µg/µg) in dimethyl sulfoxide and reading the optical density of the resulting solution at 550 nm with a microplate reader (MR 5000; Dynatech Laboratories, Embrunich-Embraport, Switzerland). Cytotoxicity was expressed as a percentage of the Teflon negative control. Differences between groups were determined by ANOVA and Tukey multiple comparison intervals (α = 0.05).

**Leakage study**

Twenty extracted human canines and premolars (n = 5 per sealer) without excessive root curvature (canal curvature 15-35°) were selected for this study. Each crown was sectioned below the cemento-enamel junction to obtain a 10-mm long root that was then prepared for endodontic treatment. During endodontic procedures, the canal space was mechanically enlarged using the Protaper endodontic files (Maillefer, Ballaigues, Switzerland) operated at 600 r.p.m. under a constant irrigation with 3% NaOCl (10 ml). The final preparation had a 6° taper and a diameter of 0.3 mm at the apex. The canals were then rinsed with distilled water (10 ml), dried with ethanol and paper points, and obturated with acrylic cones of size and taper corresponding to the canal preparation (lot #0202BF; Produits Dentaires, Vevey, Switzerland) and sealer. Acrylic cones were used because pilot studies showed that gutta-percha cones are auto-fluorescent when observed under the confocal microscope (CLSM).

**Etramethylene-bis(thioether)ane (TRITC)-labeled LPS** was used as a marker for the leakage test. Briefly, stock solutions of TRITC-LPS were prepared by mixing 1.5 mg of TRITC-LPS (Sigma, Buchs, Switzerland) with double-distilled water so that the final concentration of the solution was 0.01 mg ml⁻¹. Nail varnish was applied to the external part of the root to avoid the penetration of the tracer through dentin. The roots were then immersed into the TRITC-LPS solution for 48 h at 37°C. In some, the roots were protected from light to prevent bleaching of the rhodamine tracer.

After 48 h, all specimens were rinsed with distilled water and embedded into cold curing epoxy (Epoxy Struers, Brünen, Switzerland). The specimens were then attached to the grips of a low-speed saw (Isomet, Buehler, Lake Bluff, IL, USA) and sectioned perpendicular to the
tooth axis into 0.5-mm thick slabs. Each slab was mounted on microscopy glass slab and stored in the dark until observation under the CLSM.

The sections were observed under a CLSM (LSM 510; Carl Zeiss, Jena, Germany) with two He and Ne lasers with excitation wavelengths of 543 nm and 633 nm, respectively. The presence of the TRICL-LPS inside the canal was detected with a HeNe green laser at 543 nm. Care was taken to keep the acquisition parameters (e.g. laser beam, pinhole size, scan speed, and pixel size) constant when comparing the different specimens.

Digital images of 564 x 564 pixels were recorded using a speed of 1.69 s per scan. For analysis, images were imported into Adobe Photoshop (San Jose, CA, USA), viewed at 200% magnification, and the intensity of the rhodamine staining was viewed using the histogram function. For each image, the red intensity (representing LPS penetration) was viewed on the external root (maximum intensity), the center of the acrylic cone (minimum intensity), and at the dentin-sealer interface. The relative intensity of LPS-rhodamine leakage was estimated by dividing the intensity at the interface by the difference between the external intensity and that in the center of the cone. In this manner, each tooth served as its own control for intensity of red color. At the interface, the most intense color was selected for analysis on the basis that the worst area of LPS leakage was considered to be the most clinically relevant.

The relative LPS penetration was averaged for five specimens for each type of material at three levels of sections: < 1 mm, 1-2 mm, and 2-3 mm from the apex. The mean LPS penetration for the three levels were compared by ANOVA and Tukey post hoc comparisons (p = 0.05). To compare overall performance of the materials, the data for LPS penetration at all three levels were averaged (n = 15 for each material) and compared with ANOVA/Tukey analysis. The pooling of the data at different levels was considered an acceptable strategy, because there were no statistical differences among the levels in the first analysis.

Results

Cytotoxicity

For materials tested immediately after mixing (fresh condition), only the Roecko Seal (RSA) exhibited low cytotoxicity (high SDH activity, Fig. 1). At 24 h, the RSA was statistically equivalent to the Teflon controls (ANOVA, Tukey, p = 0.05). The Pulp canal sealer (PCS), Top Seal (TS), and Endo REZ (REZ) all suppressed SDH activity by > 60% relative to the Teflon negative controls, and all were statistically more cytotoxic that the RSA at all time-points. All materials became more cytotoxic with time of exposure to cell culture medium. The RSA, which showed little SDH suppression in the initial 24 h of testing, suppressed SDH activity of the Balb cells by as much as 35% after 1 wk. The PCS, TS, and REZ also became more cytotoxic with time, and all suppressed SDH activity by > 90% after 1 wk.

When the materials were allowed to set for 24 h before testing (set condition), the results were similar to the fresh condition at 24 h, but all tended to be more stable, and there were few statistically significant increases in SDH suppression with time (Fig. 1). One exception was the REZ, which showed complete suppression of SDH activity at 48 h. The RSA showed some tendency to increased SDH suppression with time, although differences were not statistically significant.

Sealing

The confocal technique was useful in measuring the sealing ability of the root canal sealers. On average, all materials showed significant leakage, but there was large variation among teeth (Fig. 2). For each material, some teeth were nearly free of LPS leakage, but LPS-rhodamine dye was detectable in nearly every tooth to some level, regardless of material (Fig. 3). Trends of leakage of LPS as a function of distance from the apex were not consistently demonstrable for any material or tooth (Fig. 2). Overall, the RSA material was more effective in sealing root canals against LPS leakage than any other material (Fig. 2, statistically significant, ANOVA, Tukey, p = 0.05).
Discussion

The biocompatibility of endodontic sealers is of particular importance because these materials are in direct contact with the periapical tissues and any degradation products might elicit adverse effects (27). The results of the current study show that all classes of currently available endodontic sealers pose significant biological risks, particularly in the freshly mixed condition (Fig. 1). When first mixed, all the materials except the RSA were severely cytotoxic, and all, including RSA, increased in cytotoxicity with time. The tendency to increasing cytotoxicity with time was not observed for materials that had set for 24 h (Fig. 1). However, the freshly mixed condition is relevant to clinical use because the sealers are placed into the canal unset and must set in situ. Thus, it appears that the biological risks of sealers are relatively high compared with other dental materials currently available, and that there is much room for improvement.

Of the materials tested in the current study, RSA was the least cytotoxic (Fig. 1), and was markedly better than the other sealers, which represented the major classes of sealers currently available in clinical endodontics. This result is in agreement with previous reports (28). Although the RSA exhibited some cytotoxicity over time, its cytotoxic properties are an improvement over these other materials.

Fig. 2. Rhodamine-lipopolysaccharide (LPS) penetration inside the root canal at level A (<1 mm from the apex), level B, (1-2 mm from the apex) and level C (2-3 mm from the apex). The relative intensity of rhodamine-LPS leakage was estimated by dividing the intensity of the rhodamine at the interface between the sealer and the acrylic cone by the difference between the external intensity and that in the center of the cone. Because there were large variations among teeth, ANOVA and Tukey post hoc comparisons (α = 0.05) did not show statistical differences between the three locations from the apex. The RSA material was more effective in sealing root canals against LPS leakage than any other material (statistically significant, ANOVA, Tukey, α = 0.05).

Fig. 3. (A, E) PCS specimens: micrograph (A) shows the presence of lipopolysaccharide rhodamine-LPS at the external surface of the root (bottom left) but no rhodamine-LPS penetration (arrows); in (E) the dye is observable at the interface between the sealer and the dentin. (B, F) Top Seal specimens: confocal micrograph (B) shows the presence of LPS-lipopolysaccharide at the external surface of the root (top left) and the absence of dye penetration (arrows) at the interface between the sealer and the acrylic cone; in (F) the dye is observable in the isthmus of the root canal exhibiting a defective sealing. (C, G) RSA Rocco Seal specimens: the confocal laser scanning microscopy images show no penetration of rhodamine-labeled LPS (arrows) in (C) and the presence of dye around the acrylic cone in (G). (D, H) Endo Rez specimens: confocal micrograph (D) shows the presence of rhodamine-LPS at the external surface of the root (top left) and the absence of dye penetration (arrows) at the interface between the sealer and the acrylic cone whereas the LPS-rhodamine dye has penetrated into the porosities of the resin sealer (H).
The confocal microscopic technique used in the current study to assess LPS leakage was useful and represents an improvement over alternatives such as light microscopic viewing of basic fuchsin, methylene blue, silver nitrate, or other biologically irrelevant dyes (15). A key advantage of the confocal technique is its ability to ‘see’ below the surface and avoid polishing artifacts that overestimate the penetration of dyes (29). Furthermore, the covalent bond between the LPS and rhodamine dye was nearly ideal since the rhodamine cannot diffuse without LPS, and the rhodamine is much smaller than the LPS, so that its presence probably does not reduce LPS penetration. The controls for the confocal technique were also promising, because the current provided an internal standard for dye penetration (the external root fluorescence and the canal core). Other advantages included a high sensitivity of the fluorescence and control over many important variables, such as time of exposure, temperature, and canal preparation technique. The use of nail varnish to prevent penetration of LPS by lateral canals appeared successful because several sections (Fig. 3A–D) did not have evidence of staining. This observation is in agreement with previous reports (20, 30).

Although they are not in common clinical use, acrylic points were used to condense the sealers inside the canals because gutta-percha points fluoresce. Since gutta-percha is more deformable, successful condensation of the canals would have been better than achievable in the current study. Thus, the current study design assessed the sealing properties of the sealers and not the condensation technique.

No sealer in the current study successfully prevented LPS penetration. Furthermore, all materials were highly variable in their ability to seal the root canal (Fig. 3). These issues reaffirm the difficulties in preparing and condensing a filling in endodontics. Factors such as canal preparation, cleaning, disinfection, or anatomy of the canal system all influence the ability of the sealers to prevent LPS penetration (31). The RSA material was the most promising in this regard (Fig. 2) because leakage was less as well as less variable among teeth.

In summary, the biological risk of using an endodontic sealer is critically dependent on both the cytotoxicity of the material and its ability to seal the root canal. Ideally, a material would have minimal cytotoxicity and would allow no leakage. The RSA (representing a silicone-based class of material) was by far the most promising of the sealers tested in the current study because it had low cytotoxicity and good ability to seal the root canal. However, even the RSA material was not ideal and the current results support the need to continue to develop better materials.

References


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