Contemporary Methacrylate Resin–based Root Canal Sealers Exhibit Different Degrees of *Ex Vivo* Cytotoxicity When Cured in Their Self-cured Mode

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Abstract

The cytotoxicity of four methacrylate resin–based sealers was investigated by the 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide assay, which measures cell viability by assessing its succinate dehydrogenase activity. The sealers were polymerized in the self-cured mode to simulate the setting condition upon their extrusion into periradicular tissues. Disks were prepared from EndoREZ (Ultradent, South Jordan, UT), RealSeal (SybronEndo, Orange, CA), MetaSEAL (Parkell, Farmington, NY), and RealSeal SE (SybronEndo) together with positive and negative controls. After setting, they were placed in direct contact with rat osteosarcoma (ROS 17/2.8) cells and for 5 succeeding weeks after immersing in simulated body fluid (SBF). All sealers exhibited severe toxicity initially (week 0). EndoREZ and RealSeal remained severely toxic after five cycles of SBF immersion. Toxicity of the two self-etching resin-based sealers MetaSEAL and RealSeal SE decreased gradually over time. Transmission electron microscopy of cells exposed to RealSeal SE showed variable degrees of cell injury that reflect its toxicity status. Cells with intact mitochondria were identifiable after the sealer became noncytotoxic at week 5. *(J Endod 2009;35:225–228)*

Key Words

Cytotoxicity, methacrylate resin–based sealers, MTT assay, nonetching, self-adhesive, self-etching primer, succinate dehydrogenase

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Biocompatibility is one of the factors that influence the clinician's choice of sealers in root canal treatment (1, 2). Although root fillings are designed to be contained within the canal spaces (3), sealers may be expressed into the periradicular tissues during procedural errors or with the adoption of certain filling techniques, resulting in tissue irritation and delayed healing (4). Contemporary root canal sealers exhibit a variable degree of cytotoxicity depending on the conditions under which they were tested (5, 6). Cytotoxicity tests that examine short-term cellular responses (7) may not truly reflect the results arising from prolonged contact of the sealers with periradicular tissues (8). Only a few studies have examined the longitudinal cytotoxic behavior of root canal sealers (9–12).

Dual-curable methacrylate resin-based sealers have attracted considerable attention because of their hydrophilic characteristics that enable them to wet canal walls and penetrate dentinal tubules (13), their bondability to radicular dentin via the use of self-etching primers (14), and their potential bondability to root-filling materials (15). Recently, self-adhesive (ie, self-etching) versions of these sealers have been introduced that eliminate the use of a separate priming step (16, 17), rendering them easier and faster to use. Although resin composites may show acceptable biocompatibility when polymerized under optimal light-curing conditions (18, 19), the cytotoxicity of dual-curable resin-based sealer composites should be evaluated in the self-cured mode (20, 21) because this is how the sealers polymerize in the apical third of canal walls and within the periradicular tissues (22). Casual interpretation of the cytotoxicity results of sealer composites polymerized under different/undefined curing conditions may lead to erroneous conclusions (23). It is also anticipated that longer periods will be required (24) to evaluate the cytotoxic responses of sealers that are polymerized in the self-cured mode (25) because the reduced degree of conversion (26) may result in the slow release of toxic, incompletely polymerized resinous components (27). Thus, the objective of this study was to examine the longitudinal cytotoxic behavior of four contemporary methacrylate resin-based sealers that were polymerized in the self-cured mode without adjunctive light curing to enhance the degree of conversion of their resinous components. The null hypothesis tested was that there is no difference in the ex vivo cytotoxicity response exhibited by four methacrylate resin-based sealers over a 6-week period of immersion in a simulated body fluid.

Materials and Methods

Specimen Preparation

Four dual-curable methacrylate resin-based products were investigated: EndoREZ (Ultradent, South Jordan, UT), a nonetching sealer that does not use an adjunctive dentin adhesive; RealSeal (SybronEndo, Orange, CA), a nonetching sealer that uses a separate 2-acrylamido-2-methyl-propanesulfonic acid-containing self-etching primer for bonding to radicular dentin; MetaSEAL (Parkell, Farmington, NY), a self-etching sealer that incorporates the acidic resin monomer 4-methacryloyloxyethyltrimellitate anhydride for bonding to radicular dentin; and RealSEAL SE (SybronEndo), another

self-etching sealer that bonds to radicular dentin via the use of a polymerizable methacrylate carboxylic acid/anhydride.

The sealers were mixed under aseptic conditions and packed into sterilized Teflon molds (3 mm thick × 5 mm diameter) and covered on both sides with sterilized Mylar sheets ($n = 6$). The molds were also covered with a sterilized glass slab on each side and clamped to spread the sealers and to exclude oxygen that inhibits free radical polymerization. The setup was placed inside a dark container at 37°C and 100% humidity to enable the mixed sealers to polymerize for 72 hours in the self-cured mode. Teflon and Pulp Canal Sealer (SybronEndo) disks were used as the respective negative and positive controls. The latter was chosen as the positive control because of its pronounced ex vivo cytotoxicity (1, 12).

Cell Culture

Cytotoxicity testing was performed using a rat osteosarcoma (ROS) 17/2.8 cell line (28, 29). Well-differentiated osteoblast-like cells were used instead of mouse fibroblasts (9, 11) or human periodontal ligament fibroblasts to facilitate the examination of the differential ability of sealers to stimulate osteogenesis in an ongoing study. These cells were incubated for 7 days at 37°C in a humidified 95% air–5% CO_2 atmosphere using a F-12 growth medium (Gibco-Invitrogen, Carlsbad, CA) that was supplemented with 28 mmol/L HEPES (Calbiochem, La Jolla, CA), 1.1 mmol/L $CaCl_2$ (Allied Chemical, Moristown, NJ), 5% NuSerum (Collaborative Res, Bedford, MA), and 25 mmol/L L-glutamine and 125 U/mL penicillin-streptomycin (Gibco-Invitrogen). The cells were plated at 40,000 cells/cm^2 in 0.5 mL of growth medium in a 24-well format.

Cytotoxicity of the sealers was assessed after the initial 72-hour setting period (week 0) and for 5 succeeding weeks (weeks 1–5) (12). One specimen was placed in the center of each well and secured so that the specimen was stable (29, 30). The surface area-to-volume ratio of the specimen to medium was approximately 150 mm^2/mL (30). Between tests, the specimens were aseptically removed and rinsed twice with sterile simulated body fluid (SBF). The SBF was prepared by dissolving 136.8 mmol/L NaCl, 3.0 mmol/L KCl, 2.5 mmol/L $CaCl_2 \cdot 6H_2O$, 1.5 mmol/L $MgCl_2 \cdot 6H_2O$, 0.5 mM $Na_2SO_4 \cdot 10H_2O$, 4.2 mmol/L $NaHCO_3$, and 1.0 mmol/L $K_2HPO_4 \cdot 3H_2O$ in deionized water buffered to pH 7.4 with 0.1 mol/L Tris Base and 0.1 mol/L HCl and autoclaved. Each specimen was immersed for 4 days in 10 mL of SBF before securing in a new seeded well and incubated under the same conditions with fresh growth medium before the next assay cycle.

Cytotoxicity Testing

Cell mitochondrial function was determined by estimating succinate dehydrogenase (SDH) activity using the 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (30, 31). After the removal of the sealer disk and culture medium from each well, the cells were gently washed with 1.0 mL of phosphate-buffered saline. The wash was replaced with an MTT-succinate solution (1 mg/mL MTT and 2.0 molal disodium succinate; Sigma-Aldrich, St Louis, MO) for 60 minutes at 37°C. The reaction was then quenched, and the cells were fixed with 0.5 mL of Tris-formalin (0.2 M Tris, 4% formalin, pH 7.2).

After aspiration of the solutions, the cell monolayers were rinsed with double-distilled water. The water was completely removed. Formazan crystals produced within the cells by SDH reduction of the MTT were dissolved in situ using dimethyl sulphoxide (DMSO)-NaOH (6.25% v/v 0.1 N NaOH in DMSO). One hundred microliter aliquots of the solution were transferred from each well to a 96-well plate, and the optical densities were measured at 562 nm using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA). The optical densities of the blank solutions (DMSO-NaOH only) were subtracted from all wells. The formazan content of each well was computed as a percentage of the Teflon-negative controls. Cytotoxicity responses were rated as severe (<30%), moderate (30%–60%), slight (60%–90%), or noncytotoxic (>90%) (25).

The mean absorbencies of the wells derived from the same sealer at each time period, and their standard deviation were calculated and the results analyzed quantitatively. Because the normality and homoscedasticity assumptions of the data were violated, they were analyzed by using nonparametric statistical methods. For each material, differences in SDH activity over the six time intervals were analyzed using repeated analysis of variance on ranks. Post hoc multiple comparisons were performed by using Dunn's tests with $\alpha = 0.05$.

Transmission Electron Microscopy

To examine the extent of cell injury after the cultured cells were exposed to the resin-based sealers after varying periods of SBF immersion, six freshly prepared disks from RealSeal SE were secured in seeded 24-well plates and incubated with growth medium. Cells in contact with RealSeal SE were selected for transmission electron microscopy because this enabled us to compare the time-dependent decline in cytotoxicity of this sealer with changes in the extent of cellular injury at an ultrastructural level. After each designated period, the cells were dislodged by incubating in trypsin-EDTA (Invitrogen) for 2 minutes. After neutralizing the trypsin-EDTA with fresh growth medium, the pooled dislodged cells were centrifuged at 2,000 rpm to produce a cell pellet. The latter was fixed with Karnovsky's fixative, post fixed in 1% osmium tetroxide, dehydrated in an ascending ethanol series (30%–100%), transferred to propylene oxide, and embedded in epoxy resin. Seventy nanometer thick sections were stained with uranyl acetate and Reynold's lead citrate and examined by using a JEM-1230 TEM (JEOL, Tokyo, Japan) at 80 kV.

Results

The intensity of the dissolved formazan produced by the cultured cells after the last sealer-SBF immersion cycle (week 5) is shown in Figure 1*A*. This colorimetric assay is an indirect assessment of the vigor of mitochondria function after exposure to potentially toxic substances. The results over the entire 6-week period are collectively represented in Figure 1*B*. All sealers were severely cytotoxic when initially evaluated at 72 hours after mixing (week 0). Pulp Canal Sealer, the zinc oxide eugenol–based positive control,

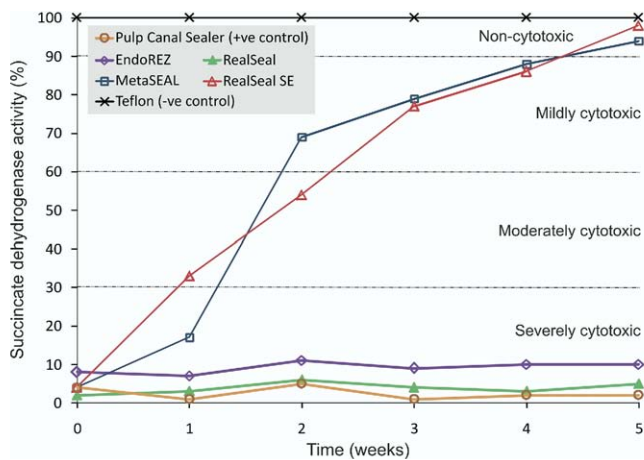

Figure 1. A line chart depicting the changes in succinate dehydrogenase activities with time after disks created from the four methacrylate resin–based sealers; the zinc oxide–eugenol–based sealer (positive control) and Teflon (negative control) were repeatedly immersed (weeks 1–5) in a simulated body fluid. Values are expressed as percentages relative to the Teflon-negative controls (100%) and are classified as severe (<30%), moderate (30%–60%), slight (60%–90%), or noncytotoxic (>90%).

TABLE 1. Succinate Dehydrogenase Activities Exhibited by ROS 17/2.8 Cells in the Presence of Different Root Canal Sealers

Materials	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
Teflon (Negative Control)	100^{A}	100^{A}	100^{A}	100^{A}	100^{A}	100^{A}
EndoREZ	7.8^{A} (2.3)	7.0^{A} (1.9)	11.4^{A} (3.5)	9.2^{A} (1.7)	10.6^{A} (2.8)	9.9^{A} (1.3)
MetaSEAL	4.0^{A} (1.3)	17.2^{B} (3.3)	69.3^{C} (9.5)	79.1^{C} (6.2)	$88.6^{C,D}$ (9.9)	94.3^{D} (3.6)
RealSeal	2.1^{A} (1.5)	3.5^{A} (1.2)	5.9^{A} (2.8)	4.2^{A} (2.0)	3.1^{A} (1.7)	5.2^{A} (2.1)
RealSeal SE	3.9^{A} (10.3)	33.3^{B} (5.6)	54.5^{B} (4.0)	77.0^{C} (6.8)	$86.1^{C,D}$ (7.4)	98.1^{D} (6.0)
Pulp Canal Sealer (Positive Control)	4.1^{A} (2.1)	1.8^{A} (0.8)	5.1^{A} (2.7)	1.7^{A} (0.7)	2.2^{A} (1.0)	2.8^{A} (0.7)

The data are normalized against the Teflon-negative control. Values represent means (standard deviations) ($n = 6$) and are expressed as relative percentages of the SDH activities of the Teflon-negative control (100%). Data derived from each sealer over different time periods are expressed in one row and analyzed separately using one-way repeated analysis of variance on ranks and Dunn's multiple comparison tests. For each row, data with different letter superscripts denotes a significant difference ($p < 0.05$).

and two methacrylate resin-based sealers, EndoREZ and RealSeal, remained severely cytotoxic over the entire experimental period (Table 1). For each of the aforementioned sealers, there was no significant decrease in toxicity ($p > 0.05$) with time. The intensities of the colored formazan in these two methacrylate resin-based sealers were similar to the Pulp Canal Sealer–positive control (Fig. 1*A*).

Conversely, the two self-etching methacrylate resin–based sealers exhibited significant increases in the SDH activities over time ($p < 0.05$, Table 1). MetaSEAL was severely cytotoxic at week 1, mildly cytotoxic at weeks 2 to 4, and became noncytotoxic at week 5. RealSeal SE was moderately cytotoxic during weeks 1 to 2, mildly cytotoxic at weeks 3 to 4, and was rendered noncytotoxic after the fifth SBF immersion cycle (Fig. 1*B*). The intensities of the colored formazan in these two sealers at week 5 were similar to the Teflon-negative control (Fig. 1*A*).

Transmission electron micrographs of the ROS 17/2.8 cells exposed to the RealSeal SE sealer after different cycles of SBF immersion showed variable degrees of cell injury that are related to the changes in cytotoxicity status of the endodontic sealer with time (Fig. 2).

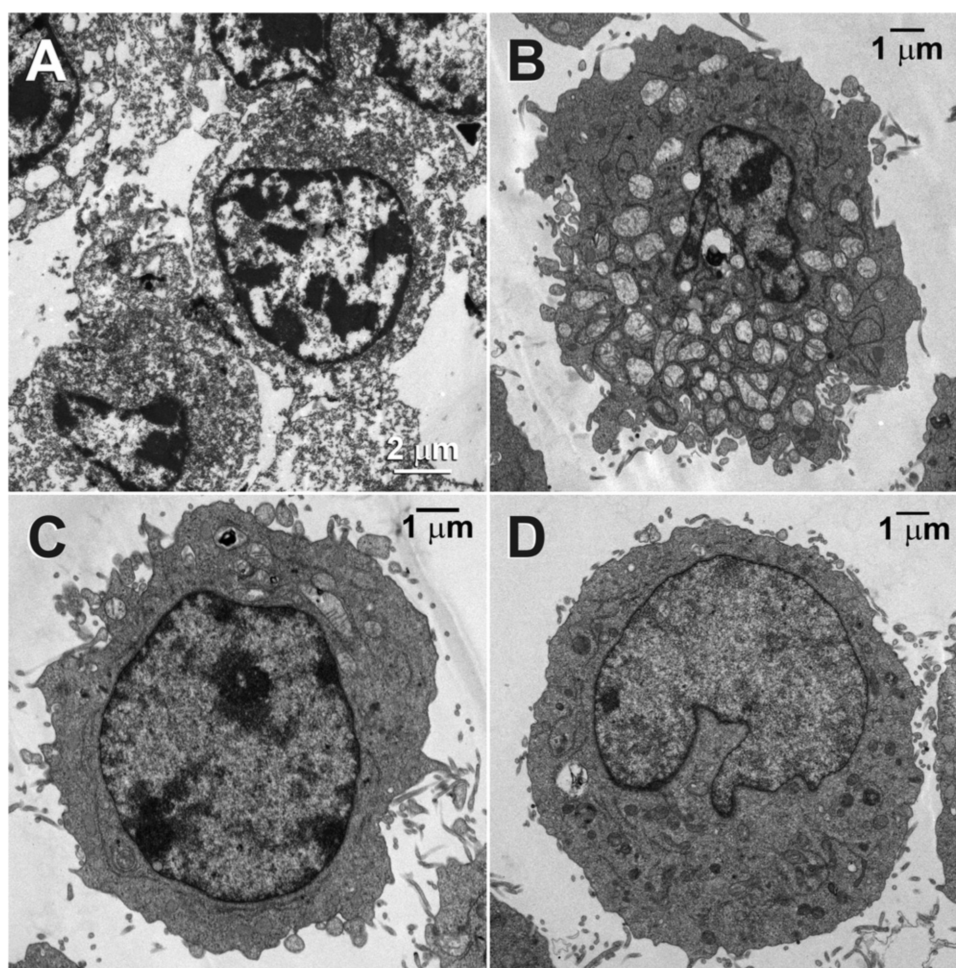

Figure 2. Transmission electron micrographs depicting the ultrastructural features of ROS 17/2.8 cells after they were exposed to RealSeal SE that had been immersed in simulated body fluid for (*A*) 0 weeks (ie, no immersion and sealer extremely cytotoxic). The cells exhibited features of necrotic cell death with condensation of nuclear chromatin and disintegration of all cellular organelles and cell membranes. (*B*) At 2 weeks (sealer moderately cytotoxic), the cell exhibited nuclear chromatin condensation and cell blebbing. Most of the mitochondria were severely swollen. (*C*) At 4 weeks (sealer mildly cytotoxic), the ultrastructure of the nucleus was normal. Within the cytoplasm, only minor swelling of some mitochondria could be discerned, and (*D*) at 5 weeks (sealer noncytotoxic), a cell with normal nucleus and cytoplasmic organelles was seen.

Discussion

The strategy of evaluating sealer cytotoxicity for extended time periods after setting was superior to previous strategies that assessed cytotoxicity for shorter time periods in that the former protocol enables the establishment of distinct toxicity profiles, which is characteristic of each sealer (9, 10, 12). Because the four resin-based sealers all elicited initially severely cytotoxic responses but showed different degrees of toxicity reduction after repeated cycles of SBF immersion, the null hypothesis that there is no difference in the ex vivo cytotoxicity response exhibited by four methacrylate resin-based sealers over a 6-week period of immersion in a simulated body fluid has to be rejected.

The relatively severe cytotoxicity responses exhibited by the two nonetching, urethane dimethacrylate–containing sealers, EndoREZ and RealSeal, confirm the results reported in previous studies (30, 32). Although only the RealSeal sealant was examined in this study, the primer component of this system has been also reported to be very cytotoxic (30). It is pertinent to note that the recently introduced self-etching methacrylate resin–based sealers (MetaSEAL and RealSeal SE) are considerably more biocompatible than the nonetching sealers. This may be because of the incorporation of a relatively high concentration of polymerizable methacrylate carboxylic acid anhydride such as 4-methacryloyloxyethyltrimellitate anhydride in these self-etching sealers. A recent study showed that a 4-methacryloyloxyethyltrimellitate anhydride-containing root-end–filling material (C&B Metabond, Parkell) also exhibited superior ex vivo compatibility in the presence of another type of bone-forming cell (33).

In conclusion, the two recently introduced third-generation self-etching methacrylate resin-based sealers (MetaSEAL and RealSeal SE) are considerably less cytotoxic than the first-generation nonetching resin-based sealer EndoREZ and the second-generation resin-based sealer RealSeal that requires the use of a separate priming step. Compared with the use of the root-dipping growth medium extracts, assays that involve direct contact of sealer disks with cultured cells are considerably more taxing because they simulate overfilling conditions instead of diffusion of sealers that are confined within root canal spaces (34). This probably explains why Pulp Canal Sealer, the positive control, exhibited consistently severe cytotoxic responses despite its clinically acceptable treatment outcome (35). Thus, the results of this study represent the worst scenario in terms of the potential biologic effects of methacrylate resin-based sealers. Although many dental materials elicit cytotoxic responses, this does not necessarily reflect the long-term risk for adverse effects because oral soft tissues are generally more resistant to toxic substances than a cell culture (25). Collectively, ex vivo and in vivo data should provide the best assessment of the overall biocompatibility of this new class of methacrylate resin–based root canal sealers.

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