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

Jason M. Ames, DMD,* Robert J. Loushine, DDS,* Brian R. Babb, DMD,* Thomas E. Bryan, BS,* Petra E. Lockwood,† Mai Sui, DDS,‡ Steven Roberts, DDS,* R. Norman Weller, DMD, MS,* David H. Pasbley, DMD, PhD,‡ and Franklin R. Tay, BDSc (Hons), PbD*†

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 periapical tissues. 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

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

From the *Department of Endodontics, School of Dentistry, Medical College of Georgia, Augusta, GA; †Department of Oral Biology, School of Dentistry, Medical College of Georgia, Augusta, GA; and ‡Guanghua School of Stomatologial & Institute of Stomatological Research, Sun Yat-sen University, Guangzhou, China.

Address requests for reprints to Dr Franklin R. Tay, Department of Endodontics, School of Dentistry, Medical College of Georgia, Augusta, GA 30912-1129. E-mail address: ftay@mcc.edu.

0099-2399/0 - see front matter
© 2008 Published by Elsevier Inc. on behalf of the American Association of Endodontists.
doi:10.1016/j.joen.2008.11.008
self-etching sealer that bonds to radicular dentin via the use of a poly-
merizable methacrylate carboxylic acid/anhydride.

The sealers were mixed under aseptic conditions and packed into
sterilized Teflon molds (3 mm thick x 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 polymeriza-
tion. 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 cyto-
toxicity (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 fibro-
blasts to facilitate the evaluation 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% CO2 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 CaCl2
(Ali-
chemical, Morristown, 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/cm2 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 mm2/mL (30). Be-


tween tests, the specimens were aseptically removed and rinsed twice
with sterile simulated body fluid (SBF). The SBF was prepared by dis-
solving 136.8 mmol/L NaCl, 3.0 mmol/L KCl, 2.5 mmol/L CaCl2
(Al-

chemical), 1.5 mmol/L MgCl2-6H2O, 0.5 mM Na2SO4, 10H2O, 4.2 mmol/L NaHCO3,
and 1.0 mmol/L K2HPO4-3H2O in deionized water buffered to pH 7.4
with 0.1 mol/L Tris Base and 0.1 mol/L HCl and autoclaved. Each spec-
imen 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 succi-
nate 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
molar 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 sulfoxide (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
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 homosce-
dasticity 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 α = 0.05.

Transmission Electron Microscopy

To examine the extent of cell injury after the cultured cells were ex-
posed 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, To-
kyo, 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 1A.
This colorimetric assay is an indirect assessment of the vigor of mitochon-
dria function after exposure to potentially toxic substances. The results over
the entire 6-week period are collectively represented in Figure 1B. All seal-
ers were severely cytotoxic when initially evaluated at 72 hours after mixing
(with week 0). Pulp Canal Sealer, the zinc oxide eugenol–based positive control,
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. 1A).

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 nontoxic at week 5. RealSeal SE was moderately cytotoxic during weeks 1 to 2, mildly cytotoxic at weeks 3 to 4, and was rendered nontoxic after the fifth SBF immersion cycle (Fig. 1B). The intensities of the colored formazan in these two sealers at week 5 were similar to the Teflon-negative control (Fig. 1A).

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).

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

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

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 ($p < 0.05$).

**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 nontoxic), 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 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.

Acknowledgments

The EndoREZ and MetaSEAL kits used in this study were generous gifts from Ultradent Products Inc and Parkell Inc, respectively. This study was supported by the Dental Research Center, School of Dentistry, Medical College of Georgia. The authors are grateful to Michelle Barnes for her secretarial support.

References