doi:10.1111/j.1365-2591.2008.01471.x

Cytotoxicity of endodontic materials over 6-weeks *ex vivo*

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Abstract

Brackett MG, Marshall A, Lockwood PE, Lewis JB, Messer RLW, Bouillaguet S, Wataha JC. Cytotoxicity of endodontic materials over 6-weeks *ex vivo*. *International Endodontic Journal*, **41**, 1072–1078, 2008.

Aim To test the hypothesis that extending the time of a traditional *ex vivo* cytotoxicity test helps to identify trends in the behaviour of root core materials and sealers, which could ultimately aid in predicting their clinical safety and performance.

Methodology Endodontic sealers and core specimens were initially tested in direct contact with L929 fibroblasts for 72 h. Cell response was estimated by measuring cellular succinate dehydrogenase activity relative to Teflon controls. Cytotoxicity (% of more active cells) was reassessed after 1, 3, 4 and 6 weeks, with the specimens stored in a physiologically balanced salt-solution between tests.

Results Distinct trends in cytotoxicity among both core materials and sealers were observed over the 6-week test. Four of the six sealers and two of the three core materials showed cell viabilities of <30% of Teflon after 6 weeks (>70% cytotoxicity).

Conclusions The current results suggest that some endodontic materials have an elevated biological risk for extended intervals.

Keywords: cell-culture, MTT, periapical inflammation, polymers, pulpal infection, succinate dehydrogenase.

Received 18 March 2008; accepted 9 August 2008

Introduction

Pulpal infections may be treated by removing diseased tissues, disinfecting the canal spaces, then filling the canal spaces with a core material and sealer to prevent fluid leakage and bacterial ingress. Although endodontic sealers are intended to be contained within the root canal, they are sometimes extruded through the apical foramina during placement (Pommel & Camps 2001). Even in the absence of extrusion, these materials often directly contact adjacent periradicular tissues (Ørstavik *et al.* 1987, Waltimo *et al.* 2001). The long-term responses of the periradicular tissues to cytotoxic materials may retard periapical healing and contribute to failure of endodontic treatment (Sjögren *et al.* 1990). Thus, the biocompatibility and sealing ability of endodontic sealers and core materials are critical to the success of root canal treatment.

Root canals are commonly filled with gutta-percha and an endodontic sealer based on calcium hydroxide, zinc oxide–eugenol, diketone, polydimethylsiloxane, glass ionomer, or epoxy-resin. Gutta-percha is commonly regarded as a nontoxic material; however, several types of gutta-percha points manufactured for endodontic use have been reported to cause cytotoxicity *ex vivo* and *in vivo* (Pascon & Spangberg 1990, Tavares *et al.* 1994, Sjögren *et al.* 1995). Conventional endodontic sealers have also been reported to be cytotoxic *ex vivo*, depending on the conditions under which testing was conducted (Miletić *et al.* 2000,

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2005, Schwarze *et al.* 2002, Öztan *et al.* 2003, Bouillaguet *et al.* 2004).

New endodontic filling strategies employ resin polymers as substitutes for traditional gutta-percha cores to fill the root canal space. Resilon (Pentron Clinical Technologies LLC, Wallingford, CT, USA) is a synthetic thermoplastic polymer based on polyester (a mixture of poly-caprolactone and urethane dimethacrylate), inorganic bioglass, bismuth oxychloride, calcium sulphate and colouring pigments (Shipper *et al.* 2004). Poly-caprolactone, a synthetic, semi-crystalline aliphatic polyester [poly(-hydroxyalkanoate)] is biodegradable and resorbable and used for medical and drug delivery devices (Pitt *et al.* 1981, Elzubair *et al.* 2006). Resilon is typically used with methacrylate resin-based sealers. These types of materials offer the possibility of bonding to both root dentine and the core material to improve sealing and create a so-called 'monoblock' obturation (Tay & Pashley 2007). Short-term moderate to severe cytotoxicity of these materials has been reported (Bouillaguet *et al.* 2006, Susini *et al.* 2006, Eldeniz *et al.* 2007, Merdad *et al.* 2007, Lodiene *et al.* 2008). Toxicity likely stems from the elution of monomers from the resin matrices (Tay *et al.* 2005, Eick *et al.* 2006, Donnelly *et al.* 2007); however, the full biological properties of these newer materials are unknown.

Ex vivo cytotoxicity tests have been extensively used to screen the biological responses to the large number of new endodontic sealers that have been introduced in the past decade (Geurtsen & Leyhausen 1997). However, even for traditional filling materials, most *ex vivo* cytotoxicity tests have been structured to expose cell cultures to the filling material only for 1 h to 14 days (Bouillaguet *et al.* 2004, 2006, Susini *et al.* 2006, Merdad *et al.* 2007). These intervals are probably inadequate to predict biological responses of materials that remain in direct contact with periradicular tissues for decades (Wataha *et al.* 1992, 1994, Hanks *et al.* 1996, Cavalcanti *et al.* 2005). For the newest materials, even short-term (24–72 h) cytotoxicity data are not available.

The objective of the current study was to assess the *ex vivo* cytotoxicity of contemporary endodontic sealers and core materials over longer time periods than have been previous reported. The hypothesis tested was that these longer-term tests provide a clearer perspective of cytotoxic behaviour. This type of information could be useful in predicting the long-term clinical performance of these materials and in formulating other biological tests to fully assess biocompatibility.

Materials and methods

Specimen preparation

Sealers and root core materials commonly used in endodontic treatment were AH Plus (Dentsply Caulk, Milford, DE, USA), Pulp Canal Sealer (Sybron Dental Specialties, Orange, CA, USA), GuttaFlow (Coltène Whaledent AG, Altstätten, Switzerland), InnoEndo (Heraeus Kulzer Inc., Armonk, NY, USA), Real Seal (Sybron Dental Specialties) and Epiphany (Pentron Clinical Technologies). The core materials investigated were gutta-percha (Obtura, Spartan, Fenton, MO, USA), Resilon (Pentron Clinical Technologies), and poly-caprolactone (Tone, The Dow Chemical Co., Midland, MI, USA). The composition of these materials and their lot numbers are summarized in Table 1.

For each material, specimens were prepared (5.5 mm in diameter and 3.0 mm in thickness $n = 4$) in sterile Teflon moulds under aseptic conditions and following manufacturer's directions. Preliminary studies established that four replicates provided sufficient statistical power to detect changes in toxicity of 10–15% given the magnitude of standard deviations when $\alpha = 0.05$. The mixed sealers were dispensed in slight excess into circular Teflon wells resting on a Mylar sheet (DuPont Teijin Films, Hopewell, VA, USA). The wells were covered with a second Mylar sheet and glass slab and clamped to spread the sealers and to restrict air from the methacrylate resin-based sealers (Nielsen *et al.* 2006). Light-curable sealers were polymerized through the glass slab for 2 min from each side using a light-curing unit at 600 mW cm^{-2} (Optilux 500; Demetron Research Corporation, Danbury, CT, USA). The specimens were allowed to set for 72 h at 37 °C in 100% humidity under sterile conditions. They were then tested for cytotoxicity (see below). Specimens for the core materials were prepared similar to the sealers. Poly-caprolactone, Resilon and gutta-percha pellets were softened in a microwave oven for 1 min and then condensed into the moulds.

Cytotoxicity testing

The materials were tested for cytotoxicity in direct contact with L929 fibroblasts (ATCC CCL1, NCTC clone 929) cultured in Dulbecco's Modification of Eagle's Medium (DMEM), 3% NuSerum, glutamine (2 mol L^{-1}), gentamicin (10 μg mL^{-1}), penicillin (125 units mL^{-1}), and streptomycin (125 μg mL^{-1}). All of these components were purchased from Gibco BRL (Invitrogen Corp.,

Table 1 Sealer and core materials tested for cytotoxicity ($n = 4$)

Material sealers	Lot no.	Comment
AH-plus Dentsply International, York, PA	0501001751	Epoxide paste: diepoxide, calcium tungstate, zirconium oxide, aerosil, pigment Amine paste: l-adamantane amine, N,N′-dibenzyl-5-oxa-nonandiamine-1,9, TCD-Diamine, calcium tungstate, zirconium oxide, aerosil, silicone oil.
Pulp Canal Sealer Sybron Dental Specialties Orange, CA	S-2250	Liquid: eugenol Powder: zinc oxide, staybelite resin, bismuth subcarbonate, barium sulfate, sodium borate anhydrate
GuttaFlow Coltène Whaledent, Switzerland	D-89122	Polydimethylsiloxane, gutta-percha powder, zinc oxide, zirconium dioxide, nano-silver, paraffin-based oil, hexachloroplatinic acid, silicic acid
InnoEndo Heraeus-Kulzer, Armonk, NY	40001987	Resins: Bis-GMA, UDMA, PEGDMA, EBPADMA Fillers: barium sulfate, bismuth oxychloride, calcium hydroxide, silica, silane-treated bariumboraluminosilicate glass; Dual-cured initiators: cumene hydroxyperoxide, thiosinamine, camphorquinone Stabilizer: butylated hydroxytoluene (2,6-di-tert-butyl-4-methylephenol); Pigment: Red #40 (CAS no. 25997-17-3
Real Seal Sybron Dental Specialties, Orange, CA	153981	Resins: Bis-GMA, UDMA, PEGDMA, EBPADMA Fillers: barium sulphate, bismuth oxychloride, calcium hydroxide, silica, silane-treated bariumboraluminosilicate glass (with a small amount of aluminum oxide); colouring pigment; Dual-cured initiators: cumene hydroxyperoxide, thiosinamine, camphorquinone; Stabiliser: butylated hydroxytoluene (2,6-di-tert-butyl-4-methylephenol)
Epiphany Pentron Clinical Technologies, LLCC. Wallingford, CT	149468	Resins: Bis-GMA, UDMA, PEGDMA, EBPADMA Fillers: barium sulfate, bismuth oxychloride, calcium hydroxide, silica, silane-treated bariumboraluminosilicate glass (with a small amount of aluminum oxide); coloring pigment; Dual-cured initiators: cumene hydroxyperoxide, thiosinamine, camphorquinone; Stabiliser: butylated hydroxytoluene (2,6-di-tert-butyl-4-methylephenol)
Cores		
Gutta-percha Obtura, Fenton Missouri	P-822-602	Zinc-oxide, gutta-percha, barium sulfate, colouring agent
Resilon Sybron Dental Specialties, Orange, CA	146646	Thermoplastic synthetic polymer –based (polycarprolactone). Bioactive glass, bismuth oxide, barium sulfate and colouring agent.
Tone Sigma-Aldrich, St Louis, MO	NA	Polymer of polycaprolactone

Bis-GMA, bisphenol A glycidyl methacrylate [2,2′-bis(4-(3-methacryloxy-2-hydroxy propoxy)-phenyl)-propane]; UDMA, urethane dimethacrylate; PEGDMA, polyethylene glycol dimethacrylate; EBPADMA, ethoxylated bisphenol A dimethacrylate; TEGDMA, triethylene glycol dimethacrylates.

Carlsbad, CA, USA). The cells were plated at 8000 cells cm^{-2} in 0.5 mL in 24-well format. The specimens were immediately (<1 min) added to the centre of each well and secured so that the specimen did not move (Wataha *et al.* 1992). The surface area-to-volume ratio of the specimen to medium was approximately 150 mm^2 mL^{-1} (within ISO 10993 specifications). In each 24-well plate, four Teflon specimens were used as controls. The cells were incubated for 72 h at 37 °C in 5% CO_2 and 100% relative humidity.

Cellular response

Cellular response to the materials was estimated by measuring mitochondrial succinate dehydrogenase (SDH) activity using the MTT (Thiazolyl Blue Tetrazolium Bromide, approximately 98% TLC, CAS #298-93-1) method as previously described (Wataha *et al.* 1992). Briefly, specimens and cell-culture medium were removed from each well and the remaining cells gently washed with 1.0 mL of phosphate-buffered saline (pH 7.4). The wash was replaced with an MTT-succinate solution (1 mg mL^{-1} MTT and 2.0 molal disodium succinate) for 60 min at 37 °C. After 60 min, the reaction was quenched and the cells were fixed by adding 0.5 mL of Tris-formalin solution (0.2 mol L^{-1} Tris, 4% formalin, pH 7.2) for 2–3 min, after which all solution was removed and the cell monolayer allowed to dry for 5–10 min.

After drying, the cell monolayer was washed with 1.0 mL of water. Then, the MTT-formazan intracellular reaction product was dissolved *in-situ*

(6.25% v/v 0.1 N NaOH in dimethyl sulfoxide [DMSO]). A 100 μL aliquot of the solution was transferred to a 96-well flat-bottomed tray and the optical density measured with a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) at 562 nm. The optical densities of the blank solutions (DMSO-NaOH only) were subtracted from all wells. The formazan content of each well was subsequently computed as a percentage of the Teflon controls for each cell-culture plate. Each percentage indicated cell viability based on SDH activity; cytotoxicity was therefore the viability subtracted from 100% (0.0% viability correspond to 70% cytotoxicity).

Ageing of the samples

Cytotoxicity testing was repeated after the initial 72 h setting period and then after 1, 3, 4, and 6 weeks as described above. A physiologically balanced salt solution (PBS, phosphate-buffered saline) was prepared by dissolving 136.8 mmol L^{-1}NaCl, 3.0 mmol L^{-1} KCl, 2.5 mmol L^{-1} $CaCl_2 \cdot 6H_2O$, 1.5 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 0.5 mmol L^{-1} $Na_2SO_4 \cdot 10H_2O$, 4.2 mmol L^{-1} $NaHCO_3$ and 1.0 mmol L^{-1} $K_2HPO_4 \cdot 3H_2O$ in deionized water, buffered to pH 7.4 with 0.1 mol L^{-1} Tris Base and 0.1 mol L^{-1} HCl and autoclaved. Between tests, the specimens were aseptically moved to wells containing 1.0 mL of sterile PBS and were incubated at 37 °C and 5% CO_2. Differences in cytotoxicity of the materials after different ageing times were determined by ANOVA with Tukey post-hoc analysis ($\alpha = 0.05$ for critical values). Tests were repeated to assess reproducibility.

Results

Of the six endodontic sealers investigated, four (Pulp Canal sealer, InnoEndo, Epiphany and RealSeal) remained severely cytotoxic over the entire experimental period (Fig. 1, SDH activity <30% of Teflon controls). L929 cells exposed to these materials showed no improvement in mitochondrial activity, even after ageing in PBS for 6 weeks.

Two of the sealers behaved differently from the others. GuttaFlow suppressed L929 mitochondrial activity by 80% initially (20% relative cell activity), but cell activity increased significantly over the initial 3 weeks of ageing. At this point cell viability was statistically similar to Teflon controls. (Fig. 1). However, cell activity in contact with GuttaFlow sealer decreased again by 40% at weeks 4 and 6.

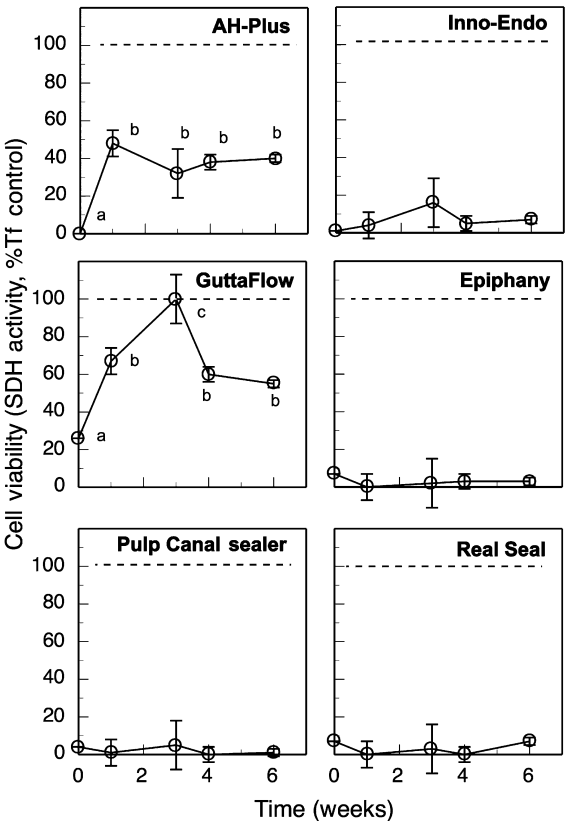

Figure 1 Cytotoxicity of endodontic sealer materials with time of ageing. Six sealers were tested in direct contact with L929 fibroblasts for 72 h initially and at 1, 3, 4 and 6 weeks, with ageing in phosphate-buffered saline (PBS) between tests. Cytotoxicity of the materials was estimated by measuring mitochondrial succinate dehydrogenase (SDH) activity and expressing activity as a percentage of Teflon negative controls (defined as 100% viability on 0% cytotoxicity, horizontal dashed line). Letters indicate statistical differences between times ($n = 4$, ANOVA, Tukey, $\alpha = 0.05$).

AH-plus was severely cytotoxic initially, but cell activity increased markedly with time, with an L929 mitochondrial activity of 50% after 1 week. Cell viability in contact with AH-plus remained at this level for the remainder of the 6 week testing period.

Both commercially available core materials (Guttapercha and Resilon) were severely cytotoxic over the entire 6 week testing period (Fig. 2). Conversely, the poly-caprolactone polymer was less cytotoxic and suppressed L929 mitochondrial activity by 40% in the first 2 weeks. Cell viability decreased to levels of the Teflon controls over weeks 3 and 4, with some relapse in week 6.

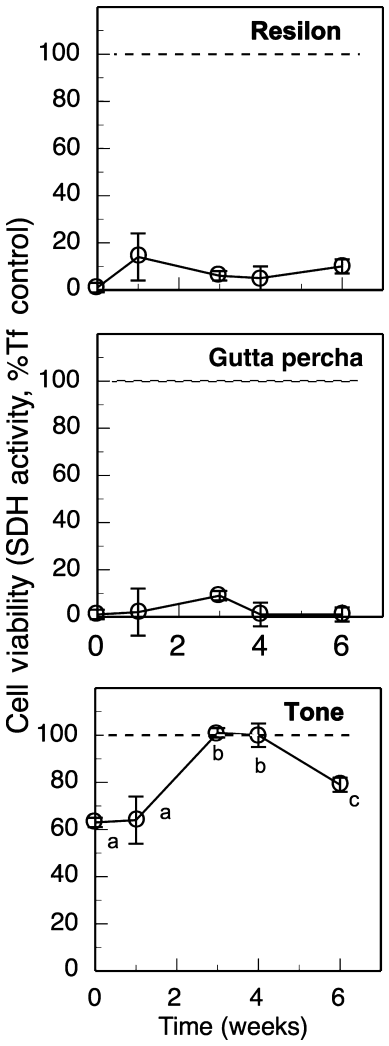

Figure 2 Cytotoxicity of core materials with time of ageing. Two commercially available core materials and poly-caprolactone were tested as described in Fig. 1. Horizontal dashed lines indicates 100% viability, (0% cytotoxicity) of the Teflon negative controls.

Discussion

Several strategies are used to evaluate *ex vivo* cytotoxicity, including direct contact and membrane restricted (diffusion) designs (Merdad *et al.* 2007, Lodiene *et al.* 2008). The current study used a direct contact design and was therefore a 'worst-case' assessment of the cytotoxic potential of these endodontic materials (Hanks *et al.* 1996). Further assessment with barrier or diffusion assays or *in vivo* assays will help better define clinical risks (Onay *et al.* 2007). The strategy of testing the cytotoxicity for extended time periods has previously been used for other materials and was superior to previous strategies that used shorter intervals (Bouillaguet *et al.* 2004, 2006, Miletić *et al.* 2005, Susini *et al.* 2006, Eldeniz *et al.* 2007, Merdad *et al.* 2007).

The current results further suggest that cytotoxicity is not a constant property, as demonstrated by the relapse in toxicity in poly-caprolactone and GuttaFlow (Figs 1, 2). In addition, the sustained cytotoxicity of several materials indicates a continued elution of material components (Table 1) at cytotoxic levels. The increase in cell activity observed for AH-plus and GuttaFlow signify a reduced biological risk *ex vivo*. Although toxicity is generally regarded as an undesirable property, the initial cytotoxicity of endodontic filling materials could be justified as an advantage whereby contaminating bacteria are killed immediately post-fill (Geurtsen & Leyhausen 1997). In support of this idea, some reports have demonstrated antibacterial activity of several of these materials (Ørstavik *et al.* 1987, Saleh *et al.* 2004).

The results of this study agree with several other that reported cytotoxic reactions to gutta-percha (Pascon & Spangberg 1990, Tavares *et al.* 1994, Sjögren *et al.* 1995). Severe cytotoxicity was observed with gutta-percha (Fig. 1) and this observation although unequivocal, is unsettling given the long-standing, broad-based use of this core material in endodontics. It is possible that differences in formulations (Table 1) may account for the inconsistent results (Pascon & Spangberg 1990). The leaching of zinc from the zinc oxide filler in most formulations also could result in cytotoxicity (Wataha *et al.* 1991, Attin *et al.* 2001).

The contrast between the severe cytotoxicity of Resilon™ (Sybron Dental Specialties, Orange, CA) (Fig. 2) and its based polymer (poly-caprolactone) was surprising (Table 1) (Pitt *et al.* 1981, Elzubair *et al.* 2006). The cytotoxicity of Resilon™ suggests that one or more of the additives to poly-caprolactone are released and cause cytotoxicity (Tay *et al.* 2005). In addition, Resilon™ has colorants that may have been a source of cytotoxicity (Table 1). The cytotoxic behaviour of AH Plus, which is an epoxy-based resin material (Fig. 1), is congruent with the corrosion behaviour of silorane resins that have been used in the formulation of some dental composites and have a similar resin chemistry (Eick *et al.* 2006). The similar cytotoxic behaviour of AH-Plus and epoxys suggests that similar corrosion processes may be occurring when the materials are placed in biological contexts

(Geurtsen & Leyhausen 1997). On the other hand, the short-term cytotoxicity of AH Plus also has been attributed to minute release of formaldehyde which decreases after setting (Leonardo *et al.* 1999).

The popularity of resin-based sealers has increased recently despite their well-documented initial cytotoxicity. A common assumption has been that the initial toxicity decreases over time (Bouillaguet *et al.* 2004, Miletić *et al.* 2005, Susini *et al.* 2006, Eldeniz *et al.* 2007, Merdad *et al.* 2007). Several authors have indicated that initial and long-term cytotoxicity of resin-based sealers are mediated by release of uncured monomers from an oxygen inhibition layer (Nielsen *et al.* 2006, Susini *et al.* 2006, Eldeniz *et al.* 2007, Merdad *et al.* 2007). However, in this study the mixed sealers were carefully prepared in an oxygen-poor environment, light-cured and allowed to set for 72 h before testing. Thus, uncured monomers seem an unlikely source of the toxicity observed in the current study. Other authors have demonstrated the solubility and degradation of these sealers when they are placed in an aqueous solution (Cavalcanti *et al.* 2005, Versiani *et al.* 2006, Donnelly *et al.* 2007).

Conclusions

With the limits of the current study, it can be concluded that an extended-repeated cytotoxicity testing strategy reveals aspects of biological behaviour that are not detected by testing strategies restricted to testing at a single time interval. Additional clinical testing should be used to confirm these *ex vivo* results.

Acknowledgements

The authors thank the Dental Research Centre of the Medical College of Georgia for financial support.

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