International Endodontic Journal

doi:10.1111/j.1365-2591.2011.01869.x

Evaluation of cytotoxic and genotoxic effects of two resin-based root-canal sealers and their components on human leucocytes *in vitro*

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Abstract

Baraba A, Želježić D, Kopjar N, Mladinić M, Anić I, Miletić I. Evaluation of cytotoxic and genotoxic effects of two resin-based root-canal sealers and their components on human leucocytes *in vitro*. *International Endodontic Journal*, **44**, 652–661, 2011.

Aim To evaluate the *in vitro* genotoxicity and cytotoxicity of two resin-based root canal sealers and to determine the type of cell death they induce.

Methodology The sealers tested were Epiphany and RealSeal. Each component of the material (Epiphany Primer, Epiphany Thinning Resin, Epiphany Sealant, RealSeal Primer, RealSeal Thinning Resin and RealSeal Root Canal Sealant), components in permutual combinations and all components mixed together were tested on human peripheral blood leucocytes using ethidium bromide/acridine orange viability staining and comet assay. Simultaneously, untreated negative control cultures were analysed in the same manner. DNA damage was evaluated following 4 h of treatment and after 24 h in the absence of the components of the materials.

Results After 4 h of treatment, except thinning resin, each individual component and the different combinations of components induced a significant increase in DNA migration ability ($P < 0.05$). After 24 h, combination of primer, thinning resin and sealant of both materials caused cell death inducing intense apoptosis. After 24 h, cells exposed to Epiphany Sealant and RealSeal Root Canal Sealant, both in polymerized and unpolymerized form, exhibited a level of DNA damage that was similar to the control.

Conclusions Primer and thinning resin of both resin-based root canal sealers and their combinations were cytotoxic and induced apoptosis. Both sealants had no significant effect on the viability of the human leucocytes.

Keywords: comet assay, cytoxicity, Epiphany, genotoxicity, RealSeal.

Received 27 November 2009; accepted 23 January 2011

Introduction

Root canal sealers frequently come in contact with periapical tissue (Waltimo *et al.* 2001), and their biological compatibility is of importance. Any irritating material extruded beyond the apical foramen may cause inflammation, delaying or preventing the healing process (Pertot *et al.* 1992). Cytotoxic materials can kill the cells in the periapex, while materials with genotoxic potential induce genome instability (Bertram 2001). Biocompatible materials should stimulate the healing of the injured tissues, without causing any adverse effects.

A large variety of materials has been used to fill root canals. Resin-based root canal sealers have favourable characteristics because of bonding to both root dentine and the core material creating a 'monoblock' (Tay & Pashley 2007). However, several studies have reported that composite resin-based sealers cause moderate to severe cytotoxicity (Bouillaguet *et al.* 2006, Susini *et al.*

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2006, Eldeniz *et al.* 2007, Merdad *et al.* 2007, Lodiene *et al.* 2008) possibly as the result of adverse effects of eluted residual monomers (Donnelly *et al.* 2007). If a material is cytotoxic, determining the type of cell death would be beneficial in estimating the biocompatibility of a material. Exposing cells to cytotoxic agents causes either necrosis or apoptosis. During necrosis, cells first swell, the plasma membrane collapses and cells are then rapidly lysed (Proskuryakov *et al.* 2003). Apoptotic cell death is generally characterized by an inward collapse of organelles, a 'blebbing' of the plasma membrane into vesicular apoptotic bodies and the destruction of genetic material (Aslan & Thomas 2009). The death and elimination of cells by apoptosis remains unnoticed by the body's immune system, while the release of the intracellular content of necrotic cells into the extracellular space induces an inflammatory response (Guimaraes & Linden 2004).

The use of genotoxicity testing is essential for evaluation of potential human toxicity so that hazards can be prevented (Ribeiro 2008). To date, a variety of assays can measure genotoxicity, such as the bacterial reverse gene mutation assay (Salmonella reversion assay or Ames test), the chromatid sister exchange, the mouse lymphoma gene mutation assay, the micronucleus test, the chromosome aberration test and the comet assay (Ribeiro 2008).

In this study, viability staining and the comet test were used for cytotoxicity and genotoxicity testing. The advantage of ethidium bromide/acridine orange viability staining is simplicity and ability to distinguish between apoptosis and necrosis based on DNA integrity. Comet assay or single-cell gel electrophoresis is a test for quantitative DNA damage assessment in mammalian cells, used for both specific DNA damage and repair detection in genotoxicity testing (Miyamae *et al.* 1998). The optimal version of the comet assay for identifying agents with genotoxic activity is the alkaline (pH > 13) version of the assay developed by Singh *et al.* (1988), which was used in the present study. This version of comet assay detects DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA-DNA/DNA protein cross-linking and SSB associated with incomplete excision repair sites. The advantages are its sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, low costs, and short time needed to complete. At alkaline pH, increased DNA migration is associated with increased levels of SSB, incomplete excision repair sites and ALS. All genotoxic agents induce more SSB and/or ALS than double-strand breaks (DSB), so this version of the assay offers greatly increased sensitivity for identifying genotoxic agents. However, DNA damage is associated with cell death, and it is critical that the highest dose tested does not induce excessive cytotoxicity (Tice *et al.* 2000). Therefore, cytotoxicity is evaluated concurrently with each genotoxicity experiment, as in the present study.

Composite resin-based root canal sealers are usually composed of few components that are used separately or mixed together. Cytotoxic and genotoxic potential of single components of composite resin-based root canal sealers or their combinations are of special importance in clinical use. Toxicity of composite resin-based root canal sealers has been evaluated using various methods and different cell types. Composite resin-based root canal sealers have been found to be moderately to highly cytotoxic in cell lines of mouse embryo cells (Balb C 3T3), mouse fibroblasts (L 929) and rat osteosarcoma cells (Lodiene *et al.* 2008, Ames *et al.* 2009, Al-Hiyasat *et al.* 2010).

The aim of this study was to evaluate and compare the *in vitro* genotoxicity and cytotoxicity of Epiphany and RealSeal using human leucocytes and to determine the type of cell death they induce, with emphasis on biocompatibility of individual components of both materials.

Materials and methods

Blood sampling

Leucocytes for evaluation of root canal sealers were obtained from three young, healthy, nonsmoking voluntary donors. Donors completed a questionnaire to confirm they had not been exposed to any physical or chemical agent that might have interfered with the results of the genotoxicity testing in the 12-month period prior to blood sampling. Blood was drawn by antecubital venipuncture into heparinized vacutainers (Becton Dickenson, Plymouth, UK). All donors signed an informed consent.

Preparation of resin-based root filling materials

In the present study, two sealers were tested: Epiphany (Pentron Clinical Technologies, Wallingford, CT, USA) and RealSeal (SybronEndo, Orange, CA, USA). Each component of the materials (Table 1), components in permutual combinations and all components mixed together were tested on human peripheral blood leucocytes using the comet assay and ethidium bromide/

Table 1 Components and composition of Epiphany and RealSeal

Epiphany	RealSeal
Epiphany Primer Champhorquinone, water, AMPS, HEMA	RealSeal Primer AMPS and hydrophilic monomers solution
Epiphany Thinning resin EBPADMA resins with photo initiator, amines, stabilizer and Red #40	RealSeal Thinning resin EBPADMA resins with photo initiator, amines, stabilizer and Red #40
Epiphany Sealant UDMA, PEGDMA, EBPADMA & BIS GMA resins, silane-treated bariumborosilicate glasses*, barium sulphate, silica, calcium hydroxide, bismuth oxychloride with amines, peroxide, photo initiator, stabilizers and pigment	RealSeal Root Canal Sealant UDMA, PEGDMA, EBPADMA & BIS GMA resins, silane-treated barriumborosilicate glasses*, barium sulphate, silica, calcium hydroxide, bismuth oxychloride with amines, peroxide, photo initiator, stabilizers and pigment
*Contains a small amount of aluminium oxide	*Contains a small amount of aluminium oxide

acridine orange viability staining. Thus, 0.02 g of each component (primer, thinning resin and sealant) was introduced in separate sterile tubes (Nange Nunc Int, Naperville, IL, USA). Epiphany Sealant and RealSeal Root Canal Sealant were tested in both polymerized and unpolymerized state. In combinatorial testing, 0.02 g of thinning resin was mixed with 0.02 g of primer and introduced in the sterile tube, and 0.02 g of thinning resin, 0.02 g of primer, and 0.02 g of sealant were mixed and introduced into another tube. Polymerization of the sealants was carried out with a halogen lamp (Elipar TriLight 3M ESPE, St Paul, MN, USA) for 40 s. Unpolymerized and polymerized sealer were only solid and had approximately 0.23 cm^2 of material surface with 5.5 mL extraction volume.

Cell treatment and recovery

Immediately after loading the tubes with root canal sealer components, 5 mL of RPMI medium (Invitrogen, Paisley, UK) was added in each tube (Greiner Bio-One, Monroe, NC, USA). pH value was checked using a pH meter (Mettler-Toledo GmbH, Schwertzenbach, Switzerland), and corrected to pH 7.2 using 10 mol L^{-1} NaOH (Sigma-Aldrich, St Louis, MO, USA). In each tube, 0.5 mL of primary leucocyte culture containing 5×10^5 cells was introduced. Leucocytes were treated for 4 h at 37 °C in 5% CO_2 atmosphere without any addition of foetal bovine serum or mitogen.

After the treatment period, cultures were centrifuged 10 min at 70 *g*, supernatant was discarded, and cells were transferred into a sterile tube. They were resuspended and the samples for vital staining and comet assay were taken. The rest of the cells were washed twice, using 5 mL of fresh RPMI medium per tube and followed by centrifugation 10 min at 70 *g*. After second centrifugation, cells were resuspended in 5 mL of RPMI and cultivated at 37 °C in 5% CO_2. After additional 24 h of incubation cultures were centrifuged, supernatant was discarded and resuspended leucocytes were sampled for evaluation of repair efficiency applying vital staining and comet assay. The same procedure of cultivation, centrifugation, washing and additional 24 h of incubation were used for negative control cultures that remained untreated. Testing was done in duplicate cultures for each component or combination of components.

Cytotoxicity testing

Leucocyte viability was tested both after 4 h of treatment and after 24 h of recovery period (Ferracane & Condon 1990, Bouillaguet *et al.* 2002, Moharamzadeh *et al.* 2009). The treatment was performed in accordance with the OECD chemical testing guidelines (OECD 1997). To detect early apoptosis and necrosis, a method described by Duke & Cohen (1992) was used. Fifty microlitres of cell culture was mixed with 50 μL of acridine orange (1 mg mL^{-1})/ethidium bromide solution (1 mg mL^{-1}), covered by cover slip and analysed under the epifluorescence microscope (AX 70; Olympus, Tokio, Japan) using 600× magnification. For each testing, 200 leucocytes were analysed in duplicate. Nuclei of vital cells emitted a green fluorescence, early apoptotic leucocytes emitted green fluorescence with condensed chromatin, late apoptotic cells red fluorescence with condensed chromatin, and necrotic red normally condensed nuclei (Fig. 1).

Comet assay

The comet assay was performed according to the standard protocol identified by Singh *et al.* (1988). Five

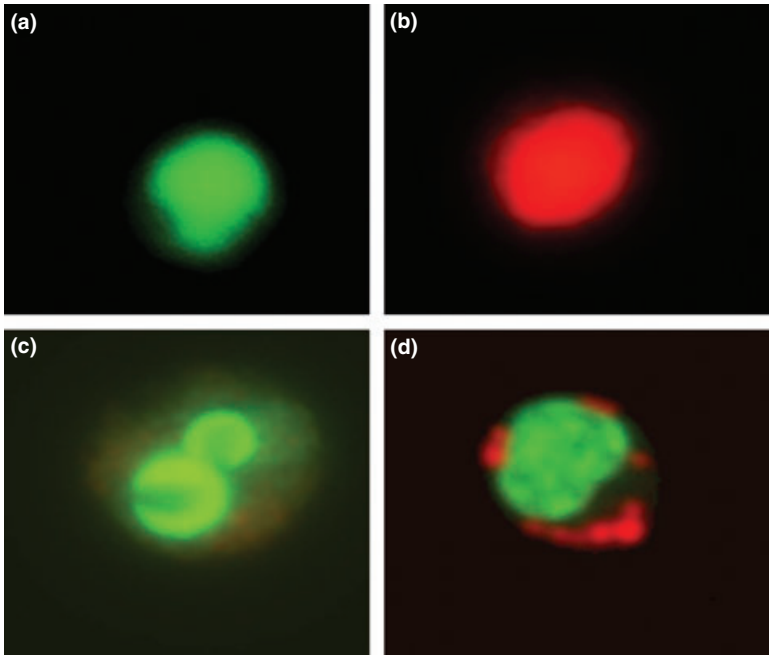

Figure 1 Appearance of leucocyte nuclei following staining with ethidium bromide and acridine orange according to the fluorescent dye exclusion method: viable normal cells excluded ethidium bromide and their nuclei were bright green with intact structure (a). Nonviable cells had orange to red coloured chromatin with organized structure (b). Apoptotic cells (c,d) were bright green with highly condensed or fragmented nuclei.

microlitres of the same cell cultures that were used for cytotoxicity testing were resuspended in 100 μL of 0.5% low melting agarose. This agarose layer was sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were coded and kept on ice during the polymerization of each gel layer. Slides were immersed in a lysis solution (1% N-lauroylsarcosine sodium salt, 2.5 mol L^{-1} NaCl, 100 mmol L^{-1} Na2EDTA, 10 mmol L^{-1} Tris–HCl, 1% Triton X-100 and DMSO 10%) at 4 °C for 1 h. Slides denaturized in electrophoresis buffer (0.3 mol L^{-1} NaOH, 1 mmol L^{-1} Na2EDTA, pH 13) at 0 °C for 20 min, and electrophoresis was carried out at 300 mA and 1.0 V/cm for 20 min. The slides were neutralized with a Tris–HCl buffer (pH 7.5) and stained with ethidium bromide (20 μg mL^{-1}) for 10 min. Each slide was analysed using a Leitz Orthoplan epifluorescence microscope (Leitz, Wetzlar, Germany) using 250 × magnification (Fig. 2). One hundred comets per slide were scored by the Comet assay II automatic digital analysis system (Perceptive Instruments Ltd, Halstead, UK) measuring tail length and tail intensity (% DNA). During analysis, the edges and eventually the damaged parts of the gel and debris, superimposed comets, comets of uniform intensity and comets without a distinct head ('clouds,' 'hedgehogs' or 'ghost cells') were avoided.

As the positive control, hydrogen peroxide (1 mmol L^{-1}) pre-treated slides were used (Collins 2004). After layering the leucocytes in agarose gel on slides, 60 μL of hydrogen peroxide (1 mmol L^{-1}) was applied for 10 min on ice. Slides were processed as described for the treated leucocytes cultures.

Statistical analysis

Kruskal–Wallis tests were used to analyse the difference between mean values of tail length and tail intensity obtained for cultures treated with Epiphany and RealSeal components and negative controls, as well as to mutually compare results obtained for the two materials. Statistical significance was set at the level of $P < 0.05$. To test significance between the cytotoxicity results for treated and control cultures, the chi-square-test was applied. Statistical analysis was performed using Statistica 7.0 (StatSoft, Tulsa, OK, USA).

Results

Apoptosis/necrosis inducing potential

After 4 and 24 h, primers and thinning resins of both materials, as well as Epiphany unpolymerized sealant, significantly decreased cell survival by mostly inducing

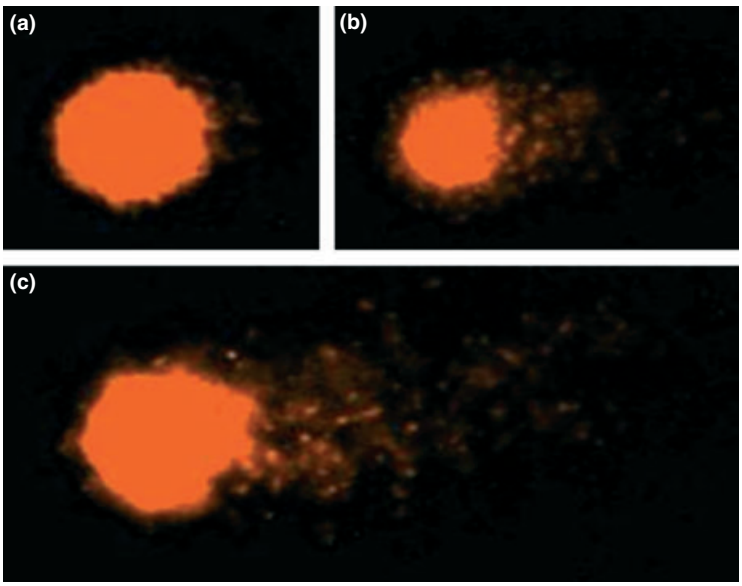

Figure 2 Comet assay microphotographs showing human leucocytes under Leitz Orthoplan epifluorescence microscope using 250× magnification (a – untreated control; b – cell treated with Epiphany; c – cell treated with hydrogen peroxide).

apoptosis ($P < 0.05$) (Table 2). Only polymerized RealSeal sealant regardless of testing period and its unpolymerized form after 24 h (Table 3) had no significant effect on the proportion of necrotic leucocytes.

In comparison with RealSeal, the combination of Epiphany primer and thinning resin and a mixture of all three components exhibited greater necrosis inducing potential ($P < 0.05$) after 4 h of treatment. After an additional 24-h recovery period, necrosis retreated to control levels ($P > 0.05$), only amongst cells exposed to polymerized Epiphany Sealant (Table 2).

Higher levels of viability were observed amongst cells treated with RealSeal components ($P < 0.05$), (Table 3). Even after a 24-h recovery period, significant proportion of cells undergoing apoptosis was observed following combined treatment with primer, thinning resin and sealant of both tested materials. The proportion of apoptotic leucocytes was significantly higher for Epiphany treatment ($P < 0.05$) (Table 2). Although apoptosis as the mechanism of induced cell death prevailed significantly, the number of cells undergoing necrosis was higher following RealSeal Root Canal Sealant treatment compared to Epiphany ($P < 0.05$). The difference in number of induced necrosis remained obvious even after 24-h recovery (Table 3).

Table 2 Cytotoxicity testing-percentage of viable, apoptotic and necrotic cells for Epiphany

Material	Procedure	Component	Living cells N ± SD	Apoptosis (early) N ± SD	Apoptosis (late) N ± SD	Necrosis N ± SD
Epiphany	4-h treatment	Primer	152 ± 4.24	39 ± 7.07	1 ± 0	8 ± 2.83
		Thinning resin*	193 ± 5.65	6 ± 6.65	0 ± 0	1 ± 0
		Primer + thinning resin*	22 ± 4.24	104 ± 14.14	37 ± 0.48	37 ± 1.41
		Unpolymerized Sealant*	131 ± 8.48	65 ± 4.24	0 ± 0	4 ± 1.41
		Primer + thinning resin + Sealant*	16 ± 2.83	149 ± 8.48	14 ± 4.24	21 ± 1.41
		Polymerized Sealant	177 ± 4.24	13 ± 1.41	2 ± 1.41	8 ± 4.24
	24-h recovery	Primer*	7 ± 2.83	24 ± 4.24	135 ± 11.31	34 ± 4.24
		Thinning resin*	2 ± 0	18 ± 1.41	159 ± 12.73	21 ± 14.14
		Primer + thinning resin*	0 ± 0	3 ± 2.83	160 ± 11.31	37 ± 8.48
		Unpolymerized Sealant*	179 ± 9.90	0 ± 0	18 ± 11.31	3 ± 1.41
		Primer + thinning resin + Sealant	0 ± 0	21 ± 5.65	164 ± 8.48	15 ± 14.14
		Polymerized Sealant	186 ± 4.24	13 ± 4.24	0 ± 0	1 ± 0
Control	Negative 4 h	/	186 ± 7.07	9 ± 1.41	0 ± 0	5 ± 5.65
	Negative 24 h	/	195 ± 5.65	4 ± 6.65	0 ± 0	1 ± 0
	Positive 4 h	/	16.7 ± 3.9	35.4 ± 12.9	29.6 ± 7.1	18.3 ± 9.5

*Statistical difference between Epiphany and RealSeal, $P < 0.05$; N, number of cells.

Table 3 Cytotoxicity testing-percentage of viable, apoptotic and necrotic cells for RealSeal

Material	Procedure	Component	Living cells N ± SD	Apoptosis (early) N ± SD	Apoptosis (late) N ± SD	Necrosis N ± SD
RealSeal	4-h treatment	Primer	135 ± 11.31	51 ± 5.65	0 ± 0	14 ± 5.65
		Thinning resin*	166 ± 8.48	27 ± 9.90	0 ± 0	7 ± 1.41
		Primer + thinning resin*	118 ± 11.31	63 ± 9.90	11 ± 4.24	8 ± 5.65
		Unpolymerized Sealant*	175 ± 9.90	19 ± 7.07	0 ± 0	6 ± 2.83
		Primer + thinning resin + Sealant	129 ± 12.73	52 ± 11.31	8 ± 4.24	11 ± 2.83
		Polymerized Sealant	184 ± 5.65	14 ± 5.65	0 ± 0	2 ± 0
	24-h recovery	Primer*	22 ± 1.41	31 ± 2.83	127 ± 4.24	20 ± 8.48
		Thinning resin*	77 ± 9.90	15 ± 1.41	95 ± 7.07	13 ± 4.24
		Primer + thinning resin*	16 ± 4.24	43 ± 5.65	132 ± 9.90	9 ± 8.48
		Unpolymerized Sealant*	185 ± 8.48	12 ± 8.48	0 ± 0	3 ± 0
		Primer + thinning resin + Sealant	2 ± 1.41	1 ± 1.41	172 ± 7.07	25 ± 7.07
		Polymerized Sealant	191 ± 8.48	7 ± 7.07	0 ± 0	2 ± 1.41
Control	Negative 4 h	/	186 ± 8.48	12 ± 8.48	0 ± 0	2 ± 0
	Negative 24 h	/	188 ± 8.48	11 ± 7.07	0 ± 0	1 ± 1.41
	Positive 4 h	/	16.7 ± 3.9	35.4 ± 12.9	29.6 ± 7.1	18.3 ± 9.5

*Statistical difference between Epiphany and RealSeal, $P < 0.05$; N, number of cells.

Genotoxicity of Epiphany and RealSeal

After 4 h of treatment, primers and unpolymerized sealants of both evaluated materials induced a significant increase in DNA migration ability ($P < 0.05$) (Tables 4 and 5). Significant increases in values of tail length and tail intensity was observed for all combined treatments. After the repair period, cells exposed to Epiphany Sealant and RealSeal Root Canal Sealant, both in polymerized and unpolymerized form, exhibited a level of DNA damage that was similar to the control values. However, primers and thinning resins, evaluated solely and in permutual combinations, induced a significant increase in both comet assay parameters ($P < 0.05$) (Tables 4 and 5). When difference between the two materials was tested, the results showed that Epiphany had higher levels of DNA damage ($P < 0.05$) for all tested components except thinning resin and polymerized sealant after 4 h of treatment (Tables 4 and 5). After the repair period, Epiphany still exhibited a higher level of DNA damage ($P < 0.05$) for all components except unpolymerized and polymerized sealant (Table 4).

Discussion

In the present study, the cytotoxic and genotoxic effects of Epiphany and RealSeal components in their single form and permutual combinations were evaluated on human leucocytes *in vitro*. Different cell cultures are commonly used for cytotoxicity and genotoxicity evaluation (Wataha *et al.* 1994, Van Wyk *et al.* 2001, Öztan *et al.* 2003). As a result of their cultivation *in vitro* for many generations, those cells undergo several genomic transformations. Thus, in studies aiming to record even a small effect on DNA level, primary cultures of isolated diploid cells, such as human leucocytes, are preferable. The normal diploid cells have mitotic rates and mitochondrial function relatively similar to *in vivo* conditions and different from those of transformed or tumour cells (Huang *et al.* 2002); thus, their response and susceptibility to xenogens will correspond more likely to those of cells *in situ*.

For comet assay, cells should be exposed to the test substance for 3–6 h (Tice *et al.* 2000). In the present study, the materials tested were placed in direct contact with leucocytes for 4 h. Direct contact between the sealer and the leucocytes simulates the clinical condition in which the sealer is extruded out of the root canal into periapical tissue, which can occur even with the best root filling technique, or simply by contact between sealer and living cells through apical foramina. Cytotoxicity and genotoxicity evaluation was performed at different time intervals, enabling the assessment of early and late toxic effects of the materials and the recovery of cells. Furthermore, the toxicity of both unpolymerized and polymerized sealants was evaluated. Local response can be provoked by unreacted and partially reacted components, and it is possible that even after the setting of the material, it still releases toxic constituents. In a study by Matsumoto *et al.* (1989), moderate and strong cytotoxicity was observed in the fresh and the set sealers. Each com-

Table 4 Comet assay-tail length and intensity for Epiphany

Material	Procedure	Component	Tail length mean (μm) ± SD	Tail intensity mean (% tail DNA) ± SD
Epiphany	4-h treatment	Primer	30.66 ± 635	12.73 ± 5.32
		Thinning resin	23.25 ± 8.66	7.62 ± 7.65
		Primer + thinning resin	101.37 ± 49.01	38.21 ± 27.69
		Unpolymerized Sealant	58.57 ± 13.82	25.36 ± 27.69
		Primer + thinning resin + Sealant	118.88 ± 31.64	43.52 ± 18.72
	24-h recovery	Polymerized Sealer	25.4 ± 5.28	9.18 ± 6.14
		Primer	144.5 ± 36.59	81.4 ± 15.85
		Thinning resin	154.87 ± 24.1	91.86 ± 6.85
		Primer + thinning resin	Cellular death	Cellular death
		Unpolymerized Sealant	24.14 ± 3.98	8.3 ± 5.14
		Primer + thinning resin + Sealant	Cellular death	Cellular death
		Polymerized Sealant	23.18 ± 3.72	6.18 ± 4.98
Control	Negative 4 h	/	23.2 ± 5.18	7.48 ± 5.82
	Negative 24 h	/	22.45 ± 4.72	6.53 ± 6.58
	Positive 4 h	/	44.3 ± 12.1	36.7 ± 26.9

Table 5 Comet assay-tail length and intensity for RealSeal

Material	Procedure	Component	Tail length mean (μm) ± SD	Tail intensity mean (% tail DNA) ± SD
RealSeal	4-h treatment	Primer	38.36 ± 17.91	11.37 ± 9.81
		Thinning resin	29.96 ± 9.81	8.33 ± 11.07
		Primer + thinning resin	44.31 ± 14.67	14.18 ± 8.95
		Unpolymerized Sealant	23.92 ± 8.64	5.13 ± 6.26
		Primer + thinning resin + Sealant	39.38 ± 13.88	16.22 ± 9.82
		Polymerized Sealer	21.35 ± 6.82	3.55 ± 5.12
	24-h recovery	Primer	106.04±12.97	89.91±4.67
		Thinning resin	88.15 ± 22.97	72.26 ± 19.86
		Primer + thinning resin	100.95±25.82	80.97 ± 23.38
		Unpolymerized Sealant	18.85 ± 8.08	1.76 ± 4.92
		Primer + thinning resin + Sealant	Cellular death	Cellular death
		Polymerized Sealant	17.2 ± 4.14	1.7 ± 3.07
Control	Negative 4 h	/	21.00 ± 6.4	7.48 ± 5.82
	Negative 24 h	/	17.96 ± 4.35	6.53 ± 6.58
	Positive 4 h	/	44.3 ± 12.1	36.7 ± 26.9

ponent of the material and their permutual combinations were tested allowing the recognition of the level of their separate or combinatorial toxicity.

All components of Epiphany and RealSeal, except polymerized sealants, induced high cytotoxic effect. The possible explanation for cytotoxicity of primers is that HEMA contained in the Epiphany primer can suppress cellular growth and cell cycle progression (Chang *et al.* 2005) or that the extractable acidic monomers in RealSeal primer are toxic (Hume & Gerzina 1996) because of their water solubility (Geurtsen 2000). The possibility of such component escaping from the confines of the root canal during application means danger for tissue destruction. Polymerized sealant, however, was not cytotoxic which is consistent with the findings that the cytotoxicity of resin-based materials varies depending upon the quantity of leachable components, which is reduced with adequate polymerization (Geurtsen 2000). This also explains the cytotoxicity of unpolymerized sealants containing UDMA, PEGDMA, EBPADMA and Bis-GMA, which have been shown to be cytotoxic (Hume & Gerzina 1996, Geurtsen 2000). Furthermore, Resende *et al.* (2009) revealed that the solubility of Epiphany was greater than values considered acceptable, allowing release of toxic substances. The high cytotoxicity of both unpolymerized resin–based sealants could be because of leaching of filler particles (Versiani *et al.*

2006) or release of unreacted monomers (Gopferisch 1996). However, the cells exposed to unpolymerized sealant recovered after 24 h, which can be explained by removal of the active components, and suggests that the observed genotoxic effect is reversible. Thinning resin was also cytotoxic, and according to the manufacturers' information, this component of Epiphany and RealSeal contains EBPADMA resins. These monomers have also been found to induce a cytotoxic response (Boland *et al.* 2006), explaining the results obtained in the present study.

To attribute additional relevance to the present study, the biological significance of the detected primary DNA damage was evaluated. Thus, following the 4 h of treatment, leucocytes were given 24-h period without the presence of tested components to determine whether they could repair the induced genome damage. Successful repair of induced lesions was observed only for leucocytes treated with the unpolymerized form of both tested sealants. These results suggest that DNA damage induced by sealants is of negligible biological importance and may not pose significant risk to the genome integrity of cells. Conversely, levels of DNA damage in leucocyte cultures treated with primers, thinning resins and their combination increased further within the 24-h recovery period. At the same time, the number of leucocytes undergoing apoptosis in the same cultures also significantly increased indicating that primary DNA damage induced by exposure to those components was too high to be repaired and it triggered programmed cell death. Thus, lesions detected by comet assay after 24 h of recovery are not the result of a genotoxic effect but of DNA fragmentation in the process of cellular death (Collins 2004). Again, the observed effect may not be directly extrapolated to the effects of endodontic material *in situ* where unreacted forms of primers and thinning resins do not remain in close contact to living tissue for long periods of time, and the ability of cells to decrease damaging effect of toxic agent significantly differ *in situ* and *ex vivo*.

The data for cytotoxicity presented here is in agreement with previous studies (Key *et al.* 2006, Eldeniz *et al.* 2007, Brackett *et al.* 2008). In a study by Key *et al.* (2006), Epiphany was tested for cytotoxicity on human fibroblasts and showed a strong cytotoxic effect. Eldeniz *et al.* (2007) found Epiphany highly cytotoxic to both human gingival fibroblasts and L929 cells, with less than 30% of viable cells after four hours treatment. Brackett *et al.* (2008) reported that both Epiphany and RealSeal remained severely cytotoxic over the period of 6 weeks on L929 cells. However, *in vitro* cytotoxicity study on human laryngeal carcinoma HEp2 cells as the model system showed that Epiphany induced necrosis as the predominant type of cell death (Brzovic *et al.* 2009a). The present study has obtained different results, with both resin-based root canal sealers inducing apoptosis in over 90% of cells. This may be explained with the different cells used for cytotoxicity testing. Tumour and normal human cells have distinct mechanisms of reacting to xenogens and this can trigger different pathways leading to either necrosis or apoptosis. Mitochondria participate in both types of cell death (Proskuryakov *et al.* 2003), so the difference in their function in tumour or normal human cells (Huang *et al.* 2002) could explain the contrary results. The study of Brzovic *et al.* (2009b) showed no genotoxic potential for Epiphany on human leucocytes, which is in accordance with the results of the present study.

Data on genotoxicity of Epiphany and RealSeal on human cells are rare. The present study found that both separate components and their combinations exhibited a genotoxic effect on human leucocytes that may be beneath biological relevance. Almost all cells were killed by both materials within 24 h, except for those exposed to polymerized sealants. Furthermore, a significant increase in induced cell death was observed even after 4 h of exposure to the materials. Thus, the observed increase in comet assay parameters was mediated by the significant cytotoxic effect of the materials, resulting in desintegration of cell compartments and fragmentation of genetic material. All those effects significantly affect the results of the comet assay. The misinterpretation of damage caused by toxicity as genotoxic lesions can be avoided by performing the cytotoxicity test as well and comparing the results of both tests.

Conclusion

Primer and thinning resin of both resin-based root canal sealers and their combinations were cytotoxic and induced apoptosis. Both sealants had no significant effect on the viability of the human leucocytes.

Acknowledgements

This investigation was supported by the Croatian Ministry of Science, Education and Sports as a part of the Themes No: 022-0222148-2137 and No: 065-0650444-0418.

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