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Cytotoxicity of new resin-, calcium hydroxide- and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines

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Abstract

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Aim To assess *ex vivo* the cytotoxic effects of five new root canal sealers (RC Sealer, Epiphany, EndoREZ, GuttaFlow and Acroseal) and three existing products (AH Plus, RoekoSeal and Apexit) using primary human gingival fibroblasts (HGF) and a mouse fibroblast cell line, L929.

Methodology Eight samples of each sealer were fabricated in sterile cylindrical Teflon blocks, 4.4 mm diameter and 2 mm height and then divided into two groups, fresh and aged specimens. Extraction of fresh specimens was carried out after setting whilst aged specimens were placed in Petri dishes and kept in a humid chamber at 37 °C for 7 days before extraction in cell culture medium using the ratio 1.25 cm^2 mL^{-1}. Undiluted eluates were used for the dimethylthiazol diphenyltetrazolium bromide (MTT) assay with HGF and L-929. Morphology of HGF cells was also examined by an inverted microscope using undiluted eluates of the sealers. The results were analysed using a two-tailed *t*-test ($\alpha = 0.05$) between groups.

Results Resin-based (Epiphany and EndoREZ) and calcium hydroxide-based (Apexit and Acroseal) sealers were significantly more cytotoxic than other sealers ($P < 0.05$). However, L929 cells were more sensitive to Apexit and EndoREZ than HGF cells. RC Sealer showed mild cytotoxicity to HGF at both setting times. AH Plus did not exert any cytotoxic effect to HGF and aged specimens appeared to induce cellular proliferation. RoekoSeal and GuttaFlow also demonstrated mild cytotoxicity. GuttaFlow was slightly more cytotoxic to both cultures, especially when tested fresh.

Conclusions Toxicity varied but RC Sealer and GuttaFlow were the least toxic new sealers.

Keywords: cell culture, cytotoxicity, root canal sealers.

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Introduction

Root canal treatment aims to eliminate infection of the root canal and to completely fill the root canal space in order to prevent apical and coronal penetration of liquids and microorganisms. Currently, most root canals are filled with gutta-percha points in combination with an endodontic sealer. The main function of the sealer is to fill the gaps between the gutta-percha points and the walls of the root canal. The sealer also fills the voids between individual gutta-percha points applied during condensation. It is widely recognized that sealers may come in direct contact with the soft and hard tissues apically for a prolonged period of time and might affect the periapical tissue, if extruded. In such a condition, they could cause not only

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degeneration of the tissue lying underneath the endodontic sealer but could also delay wound healing. Therefore, the biocompability of the sealers is of primary importance (Geurtsen 2001).

The biocompatibility of different root canal sealers varies considerably (Geurtsen 2000). Most products exert some toxic effect, when they are fresh and the effect is reduced over time as the concentration of leachable components decreases (Araki *et al.* 1994, Thom *et al.* 2003). Root canal filling materials have been formulated in an attempt to obtain better physical and biological properties (Barbosa *et al.* 1993).

Commonly applied root canal sealers have a number of bases: epoxy resin, calcium hydroxide, zinc-oxide eugenol or silicone. The popularity of resin-based sealers is increasing, despite their well-documented toxicity and mutagenicity (Schweikl *et al.* 1998, Huang *et al.* 2002a). In addition, leakage has been observed between sealer and dentinal wall as a result of contraction of the resin sealers during setting (De Almeida *et al.* 2000). Thus, new resin formulations have been designed to improve the adhesion of the sealers to dentine both in combination with a dentine primer (Epiphany) and without (EndoREZ and RC Sealer).

Calcium hydroxide-based sealers may promote hard tissue formation, but they tend to dissolve over time and may thus compromise the endodontic seal (Hovland & Dumsha 1985, Huang *et al.* 2002b). A new calcium hydroxide-based sealer, Acroseal, appears to have lower solubility than other calcium hydroxide sealers, probably because of its epoxy resin component (Eldeniz *et al.* 2007).

Silicone-based materials have been developed as root canal sealers and laboratory and clinical data are promising (Wu *et al.* 2002, Huumonen *et al.* 2003). GuttaFlow is a new silicone-based material that contains gutta-percha powder.

Cell culture techniques are useful for evaluation of the biocompatibility of medical devices (Schwarze *et al.* 2002) and also have the advantages of being an inexpensive and quick way of screening large number of materials (Vajrabhaya & Sithisarn 1997). The cytotoxicity can be determined with reliability and reproducibility (Arenholt-Bindslev & Horsted-Bindslev 1989, Beltes *et al.* 1995). The American Dental Association and the International Standards Organization Committee concerned with dentistry (ISO/TC 106 Dentistry) have also encouraged the use of *ex vivo* methods (ANSI/ADA 1979, ISO 7405 1997, ISO 10993 1992).

The dimethylthiazol diphenyltetrazolium bromide (MTT) assay measures cellular metabolic function and is widely used for *ex vivo* biocompatibility evaluation (Huang *et al.* 2002b, Camps & About 2003, Huang *et al.* 2004). The advantages of this method are its simplicity, rapidity and reliability. In addition, it does not require radioisotopes.

The aim of the current study was to investigate the cytotoxic effects of eluates of five new root canal sealers on human gingival fibroblast (HGF) cells and the established mouse fibroblast cell line, L929 and to compare the results with those of three products that have been on the market for some time.

Materials and methods

Sealers

Five new (Epiphany [Pentron Clinical Technologies LLC, Wallingford, CT, USA], EndoREZ [Ultradent Product Inc., South Jordan, UT, USA], RC Sealer [Sun Medical Co. Ltd., Furutaka-cho, Moriyama, Shiga, Japan], Acroseal [Septodont, Saint-Maur-des-Fossés, France], GuttaFlow [Roeko, Colténe/Whaledent, Langenau, Germany]) and three well established (AH Plus [Dentsply De Trey, GmbH, Konstanz, Germany], Apexit [Ivoclar-Vivadent, Schaan, Liechtenstein], RoekoSeal [Colténe/Whaledent, Langenau, Germany]) root canal sealers were evaluated. The materials tested were resin-based (Table 1), calcium hydroxide-based (Table 2) and silicone-based (Table 3).

Sample preparation

The sealers were mixed according to the manufacturers' instructions. Eight discs for each sealer were fabricated under aseptic conditions in sterile cylindrical Teflon blocks, 4.4 mm in diameter and 2 mm in height. The test specimens were placed in a humid chamber at 37 °C, thrice the length of the setting time given by the manufacturer to secure proper setting. Excess flash material was removed with a sterile scalpel. Immediately after setting, four samples from each product were immersed in extraction media (fresh specimens). Another group of the samples were placed in Petri dishes and kept in a humid chamber at 37 °C for 7 days before the extraction procedure (aged specimens).

Preparation of extracts

The extraction was made in cell culture medium using the ratio 1.25 cm^2 mL^{-1} between the surface of the samples and the volume of medium. The

Table 1 Resin-based root canal sealers tested

Material	Manufacturer	Lot no.	Ingredients	
			Paste A	Paste B
AH Plus	De Trey/Dentsply, Konstanz, Germany	0306001037	Epoxy resin Calcium tungtate Zirconium oxide Aerosil Iron oxide	Adamantane amine *N,N*-Dibenzoyl-5-oxanonane TCD-Diamine Calcium tungstate Zirconium oxide Aerosil Silicone oil
EndoREZ	Ultradent/USA	66VD	30% Urethane dimethacrylate, zinc oxide, barium sulphate, pigments	
Epiphany	Pentron, Wallingford, CT, USA	103856	BisGMA, UDMA and hydrophilic methacrylates	
RC Sealer	Sun Medical/Japan	Catalyst: KG12 Monomer: KR1 Powder: 040226	Catalyst: TBB partially oxidated Monomer: 4-META/MMA Powder: polymethylmethacrylate/ZrO_2	

Table 2 Calcium hydroxide-based root canal sealers tested

Material	Manufacturer	Lot no.	Ingredients	
			Paste A	Paste B
Acroseal	Septodont, France	For Catalyst M4 098, Base M3 190	Calcium hydroxide DGEBA Radiopaque excipient	Glycyrrhetic acid (enoxolone) Methenamine Radiopaque excipient
Apexit	Vivadent, Schaan, Liechtenstein	F65075	Calcium hydroxide Hydrogenized colophony Silicon dioxide Paraffin oil Zinc oxide Calcium oxide Polydimethysiloxane Zinc stearate Pigments	Trimethylhexandedioldisalicylate Bismuth carbonate Bismuth oxide Silicon dioxide 1,3-Butanedioldisalicylate Hydrogenized colophony Tricalciumphosphate Zinc stearate

Table 3 Silicone-based root canal sealers tested

Material	Manufacturer	Lot no.	Ingredients	
GuttaFlow	Colthane/Whaledent Langenau/Germany	6407042	Gutta-percha powder Polydimethylsiloxane Silicone oil, Paraffin oil	Hexachloroplatinic acid Zirconium oxide Nano-silver (preservative)
RoekoSeal	Colthane/Whaledent Langenau/Germany	6405904	Polymethylsiloxane, silicone oil, paraffin-base oil, hexachloroplatinic acid, zirconium dioxide	

extraction vials were agitated for 24 h in a water bath at 37 °C according to ISO Standard 10993-12. Control samples containing only medium were treated similarly. The test samples were removed and the extracts were sterile filtered using Millex-GS sterile filter (Milipore S.A.S., Molsheim, Cedex, France). Undiluted extracts were used for the testing.

Cell cultures

Human gingival fibroblasts were isolated from biopsies taken during oral surgery procedures, cultured and maintained in plastic culture flasks according to the technique described by Liu *et al.* (1991). Briefly, the cells were harvested between the fourth and eighth

passages and cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified eagle's medium (DMEM, Sigma, St Louis, MO, USA), supplemented with 5% fetal calf serum (FCS) and containing penicillin (50 IU mL^{-1}) and streptomycin (50 μg mL^{-1}) solution.

An established cell line, mouse fibroblasts L929 (American Type Culture Collection CCL 1) was cultivated in minimal essential medium (MEM) (PAA Laboratories, Pasching, Austria), supplemented with 5% fetal calf serum (Sigma–Aldrich, St Louis, MO, USA), 100 U mL^{-1} penicillin, 100 μL mL^{-1} streptomycin and 2 mmol L^{-1} L-glutamine (Cambrex Bio Science, Verviers, Belgium). Sub-cultivation was performed with cells from confluent cultures treated with 0.5 g L^{-1}/ 0.2 g L^{-1} ethylenediaminetetraaceticacid in phosphate-buffered saline (PBS).

Cells were diluted in fresh medium and seeded into 96-well plates (L929: 1.5×10^4 cells $well^{-1}$, HGF: 2×10^4 cells $well^{-1}$). After incubation for 24 h, the medium was aspirated from all wells and replaced with 100 μL $well^{-1}$ extraction or control medium and incubated for another 24 h before cytotoxicity was addressed.

Cytotoxicity assay

The colorimetric assay developed by Mosmann (1983) and modified by Edmondson *et al.* (1988) was used as a test for cell proliferation and survival in this study. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye solution (MTT) (Sigma, St Louis, MO, USA) was prepared as 5 mg mL^{-1} in PBS at 37 °C just before use. A total of 20 μL MTT dye was added to each well and incubated at 37 °C, in air containing 5% CO_2 and at 95% relative humidity for 4 h in the dark. After incubation, the MTT was aspirated and the formazan product was solubilized in 0.1 mL in HCl (0.04 mol L^{-1}) in isopropanol. The plates were shaken before the optical densities were measured at 570 nm, using a Multiskan EX spectrophotometer (Labsystem, Helsinki, Finland). Six replicates of each extract or control were performed in each test. All assays were repeated at least twice to ensure reproducibility.

Statistical analysis

The mean absorbencies of the six wells containing the same extract and their standard deviation were calculated. Original optical density values of test cultures were expressed as percentage of optical density obtained for the control medium. The absorption value obtained with the control was considered as indicating 100% viability. Cytotoxicity was also rated based on cell viability relative to controls as not cytotoxic – >90% cell viability, slightly cytotoxic – 60–90% cell viability, moderately cytotoxic – 30–59% cell viability and strongly cytotoxic – <30% cell viability (Dahl *et al.* 2006).

Statistical differences between the root canal materials and the controls were determined using a two-tailed *t*-test. The effects of different root canal sealers on cell viability were deemed significant for ($P < 0.05$). All computations were made using the SPSS 7.0 statistical software package (SPSS Inc., Chicago, IL, USA).

Cell morphology

In order to evaluate the effect of the root canal sealers on cell morphology, extracts were made of four additional samples from each product. HGF were exposed to extracts for 4 h in 6-well plates and, after staining the cells with trypan blue, cell morphology was evaluated by light microscopy (Nikon ELWD 0.3 inverted microscope at ×100 magnification).

Results

The results of the MTT assays are given in Tables 4 and 5 for the HGF and L929 cells, respectively. Extracts of four of the root canal sealers, two resin-based (Epiphany and EndoREZ) and two calcium

Table 4 Cytotoxic effect of eight root canal sealers on human gingival fibroblasts cells expressed in percentage of viable cells compared with control in fresh and aged samples. The rating of cytotoxicity for each sealer indicated in the last column

	% Cell viability*		
Sealer	Fresh samples	Aged samples	Cytotoxicity
AH Plus	94.4 ± 8.91	133.5 ± 11.25	Not cytotoxic
EndoREZ[a]	10.4 ± 4.26	9.3 ± 4.06	Strongly cytotoxic
Epiphany[a]	7 ± 3.79	7.4 ± 3.91	Strongly cytotoxic
RC Sealer[b]	88.3 ± 8.02	83.9 ± 7.82	Slightly cytotoxic
Acroseal[a]	10.2 ± 3.92	8.3 ± 3.92	Strongly cytotoxic
Apexit[a]	9.8 ± 3.55	12.4 ± 3.30	Strongly cytotoxic
GuttaFlow	63.1 ± 7.65	75.6 ± 6.04	Slightly cytotoxic
RoekoSeal[b]	79.6 ± 6.71	84.4 ± 6.37	Slightly cytotoxic

*Mean ± SD, $n = 6$.
[a,b]Same superscript letters indicate materials with characteristics that do not differ significantly when tested fresh or aged ($P > 0.05$).

Table 5 Cytotoxic effect of eight root canal sealers on L929 cells expressed in percentage of viable cells compared with control in fresh and aged samples. The rating of cytotoxicity for each sealer indicated in the last column

Sealer	% Cell viability*		Cytotoxicity
	Fresh samples	Aged samples	
AH Plus	4.9 ± 0. 8	68.9 ± 1.7	Slightly cytotoxic
EndoREZ[a]	1.2 ± 0.4	0.3 ± 0.3	Strongly cytotoxic
Epiphany[a]	1.5 ± 0.4	1.5 ± 0.3	Strongly cytotoxic
RC Sealer	29 ± 4	58.6 ± 1.3	Moderately cytotoxic
Acroseal[a]	0.7 ± 0.45	1.2 ± 0.6	Strongly cytotoxic
Apexit[a]	1.1 ± 0.5	0 ± 0.45	Strongly cytotoxic
GuttaFlow[b]	68 ± 11	76 ± 2.3	Slightly cytotoxic
RoekoSeal[b]	72 ± 11	79 ± 2	Slightly cytotoxic

*Mean ± SD, $n = 6$.
[a,b] Same superscript letters indicate materials with characteristics that do not differ significantly when tested fresh or aged ($P > 0.05$).

hydroxide-based (Apexit and Acroseal) were significantly more cytotoxic than the other sealers and this effect was the same for both fresh and aged specimens. L929 cells were generally more sensitive than HGF cells.

Morphologically, HGF of the control group and those in contact with the eluates of silicone-based sealers attached on the flat surface of culture dishes demonstrated the typical stellate appearance of this type of cell. Some cells with filopodia and laminipodia could be observed [Fig. 1(a,b)]. When extracts of the sealers Epiphany, EndoREZ, Apexit and Acroseal had been added, the cells changed their shape, becoming streaked or rounded and lost their structural organization (Fig. 1c). Most of these cells could not exclude trypan blue, implicating the presence of cell membrane damage and loss of cell viability. Cells treated with eluates of RC Sealer for 4 h demonstrated atypical morphologies (Fig. 1d).

Discussion

Although laboratory studies offer a convenient means of observing how cells interact with biomaterials, it is important to differentiate between studies using commercial cell lines and primary cultures of human cells. It has been argued that established cell lines are well suited for screening purposes and provide more reproducible results than primary cells (Groth *et al.* 1995). The L929 cells are commonly used to evaluate the cytotoxicity of root canal sealers and reported to be more prone to toxic products than HGF (Pissiotis & Spångberg 1991). Despite the popularity of the L929

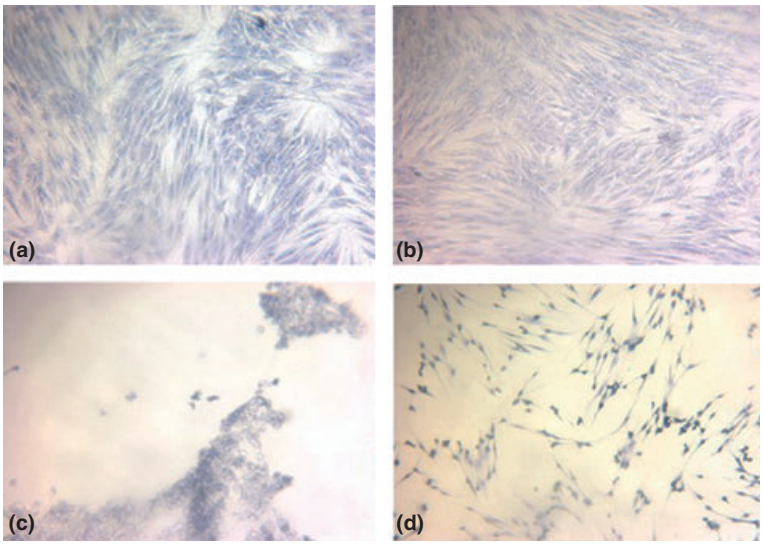

Figure 1 (a) Typical bipolar spindle-shaped morphology of normal human gingival fibroblasts, cellular filopodia and laminipodia could be observed in the control group. (b) Normal spindle-shaped morphology of human gingival fibroblasts cells after exposed to the eluates of GuttaFlow sealer. (c) Rounded or streaked shape of human gingival fibroblasts cells when treated with medium containing the eluates of Epiphany sealer for 4 h. This indicated that most cells have lost their viability. (d) Cells of human gingival fibroblasts cells showed atypical morphologies after incubation with the eluates of root canal sealer for 4 h (×100 original magnification).

cells for cytotoxicity experiments, the appropriateness of the use of such cells is often questioned. The main argument is that these cells have a heteroploid chromosome pattern and may respond differently to toxic materials than the relevant target cells in human (Kasten *et al.* 1982, Browne 1985). Furthermore, the normal diploid cells have different mitotic rate, density-dependant regulation of growth, different mitochondrial function compared with aneuploid cell lines derived from other tissues and species (Huang *et al.* 2002b) and more tolerance to toxic products (Al-Nazhan & Spångberg 1990). From a biological standpoint, the use of human oral cells derived directly from the target tissues might be more relevant to the clinical setting and therefore, the use of both HGF and L929 cells was well motivated in the present study.

AH 26 root canal sealer was the first epoxy resin-based sealer and has been in use for several decades. However, it has been demonstrated that the sealer is cytotoxic during and after setting (Gerosa *et al.* 1995, Geurtsen *et al.* 1998, Osorio *et al.* 1998) that was explained by the presence of formaldehyde released as a chemical by-product during setting (Spångberg *et al.* 1993). AH Plus was subsequently developed and according to the manufacturer this is a 'formaldehyde free' material. However, in the present study fresh specimen extracts of AH Plus significantly inhibited the growth of L929 cells and exerted a strong cytotoxic effect. This might have been caused by minute amounts of formaldehyde from the sealer or by the release of the amine and epoxy resin components of the sealer (Cohen *et al.* 1998). The result obtained in this study largely confirmed those in previous reports (Cohen *et al.* 2000, Milétic *et al.* 2000, Willershausen *et al.* 2000, Tai *et al.* 2001, Huang *et al.* 2004, Milétic *et al.* 2005). Probably as a result of the diminishment in the leaching of toxic substances, the cytotoxicty of AH Plus decreased in the aged specimens (Milétic *et al.* 2000, Azar *et al.* 2000, Huang *et al.* 2004). Contrary to the present experiment, it has been indicated that AH Plus is cytocompatible (Leyhausen *et al.* 1999, Camps & About 2003). The discrepancy between the results could be explained by variation in the conditions of the experiments.

Root canal sealer is based on methyl methacrylate/tributylborane (MMA/TBB) resin (Leonard *et al.* 1996) which is present in the resin cement material C & B Metabond (Parkell, Farmingdale, NY, USA) and in Super Bond C & B (Sun Medical Co., Shiga, Japan). Imai & Komabayashi (2003) started a project to make this material suitable for root canal filling in the early 1990s. The major problems were short working time, low radiopacity and difficulty in the removal of the resin from the root canal. These problems were solved by substituting the polymer component of the composite resin with a specially selected polymethyl methacrylate (PMMA) in the product now known as RC Sealer (Test sealer, Sun Medical Co., Ltd, Furutaka-cho, Moriyama, Shiga, Japan). It also contains partially oxidized tri-*n*-butylborane (TBBO) as a catalyst and 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate (4-META/MMA) as monomer. The data obtained in this study indicated that RC Sealer inhibited growth of cells and exerted a strong cytotoxic effect on L929 cells when extracts of fresh samples were tested and eluates of both fresh and aged specimens were slightly cytotoxic to HGF. This toxic effect may be associated with the TBBO component of this sealer (Fujisawa & Atsumi 2004).

The present study demonstrated a toxic effect for EndoREZ that did not decrease with time. The cytotoxicty of EndoREZ had been demonstrated in a previous laboratory study (Bouillaguet *et al.* 2004) and in an animal study where subcutaneous implantation of EndoREZ to the connective tissue of rats caused mild to severe tissue reactions which subsided after 30 days (Zmener 2004). Urethane dimethacrylate (UDMA) in the structure of this sealer could be responsible for the cytotoxic effect, as it has been previously shown that UDMA is a toxic agent (Hikage *et al.* 1999).

Epiphany is the root canal sealer of the Resilon system and this system is commercially available as RealSeal (SybronEndo, Orange, CA, USA) and as Next (Heraeus-Kulzer, Hanau, Germany). This sealer is composed of fillers of calcium hydroxide, barium sulphate, barium glass and silica. It is reported that the total filler content in the sealer is approximately 70% by weight (Versiani *et al.* 2006). A possible explanation for the high cytotoxicity of this sealer could be the leaching of filler particles of the sealer as a result of degradation (Versiani *et al.* 2006). It has been demonstrated that water diffusion also leads to erosion of the composite resin material causing release of unreacted monomers (Gopferisch 1996). Epiphany is a dual curable methacrylate resin sealer and based on a mixture of bisphenol A-glycidyl methacylate (Bis-GMA), ethoxylated BisGMA, urethane dimethacylate (UDMA) and hydrophilic difunctional methacylates (Versiani *et al.* 2006). Another reason for the high cytotoxicity of this sealer regardless of fresh or aged specimens could be the residual monomers which were shown to be the main components released from cured dental composite materials (Ruyter 1995).

The calcium hydroxide-based Apexit sealer proved to be highly cytotoxic in both cultures. This is not in agreement with the previous findings by others (Beltes *et al.* 1995, Vajrabhaya & Sithisarn 1997, Geurtsen *et al.* 1998, Miletić *et al.* 2000, Schwarze *et al.* 2002). This could be attributable to the differences in methodology of cytotoxicity testing [cell counting, XTT assay, 51Cr-release, Sulphorhodamine-B (SRB) dye staining methods] and/or cell line used (BHK 21/C13, HeLa, 3T3 mouse fibroblasts, human periodontal ligament fibroblasts, Mu-mu-1). Acroseal, another new calcium hydroxide-based sealer, was also found to be strongly cytotoxic to both cell lines. The source of the toxicity might be because of the presence of amines in the epoxy base of this material.

There are a few reports available in the literature on the cytotoxicity of silicone-based root canal filling materials (Briseño & Willershausen 1991, Öztan *et al.* 2003, Al-Awadhi *et al.* 2004, Bouillaguet *et al.* 2004, Milétic *et al.* 2005). Silicone-based sealers, both fresh and aged specimens, demonstrated slight cytotoxic effects on both cultures in the present study. This is in accordance with other results reported in previous studies (Schwarze *et al.* 2002, Bouillaguet *et al.* 2004, Milétic *et al.* 2005). GuttaFlow was slightly more cytotoxic than RoekoSeal to both cell cultures in the present study. This could be because of some extra additives in the content of GuttaFlow. It contains gutta-percha powder and nano-silver as a preservative. In a previous study by Sjögren *et al.* (1995) the tissue reaction to fine gutta-percha particles was demonstrated. It was also demonstrated that nano-silver-based inorganic antibacterial agents had a variable cytotoxic effect on mouse fibroblasts L929 depending on the concentration used (Zhang *et al.* 2005).

Conclusion

The new RC sealer and GuttaFlow sealers were less cytotoxic than the other three new sealers tested (Epiphany, EndoREZ and Acroseal). Toxicities were generally of a magnitude similar to that of established types of root canal sealers. These new sealers require further investigations into other properties essential for successful root canal treatment.

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