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# Comparative study of biocompatibility of newly developed calcium phosphate-based root canal sealers on fibroblasts derived from primary human gingiva and a mouse L929 cell line

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## Abstract

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**Aim** To determine biocompatibility of three calcium phosphate cement (CPC) sealers, and to compare the cytotoxic response of human gingival fibroblasts (HGF) and one mouse fibroblast cell line (L929) to these materials.

**Methodology** Monocalcium phosphate, calcium oxide and synthetic hydroxyapatite were combined with one of three aqueous solutions: modified polyacrylic acid, glass–ionomer liquid or 35% w/w polymethyl vinyl ether maleic acid to obtain Types I, IIa and III CPCs, respectively. Commercial $Ca(OH)^2$ sealer was used as a control. The materials were packed into Teflon molds (5.5 × 3 mm), and cellular function was assessed using MTT assay. The specimens were placed immediately in contact with cells, then evaluated at (24 h, 1 week, 2 week, 3 week, 4 week, 5 week).

**Results** All materials showed significant cytotoxicity for both L929 and HGF cells at 24 h except for Type III. Type I was severely toxic initially, but improved significantly ($P < 0.05$) over the 5 week evaluation. Types II and $Ca(OH)^2$ were both cytotoxic over the 5 weeks. Type III CPC was equivalent to Teflon the entire time. The results showed the same rank of cytotoxicity in both cultures. The cytotoxic response decreased in the order of Type II > $Ca(OH)^2$ > Type I > Type III overtime. L929 cells were generally more sensitive than HGF cells to the calcium hydroxide-based sealer (Acroseal).

**Conclusion** Types I and III have acceptable biologic properties for endodontic applications.

**Keywords:** calcium phosphate cements, cell culture, cytotoxicity, human gingival fibroblasts, L929 mouse fibroblast cell line.

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## Introduction

Root canal treatment aims to eliminate infection of the root canal and to completely fill the canal space in order to prevent apical and coronal penetration of liquids and microorganisms. Various methods have been proposed for root canal filling. The most frequently used methods use semisolid materials such as gutta-percha in combination with a root canal sealer or paste. Biological compatibility of root canal sealers is of importance as these materials come into contact with periapical tissues including fibroblasts. The tissue response to these materials may influence the final outcome of root canal treatment (Waltimo *et al.* 2001).

Several classes of endodontic sealers are currently used in clinical practice, but all have substantial limitations. Zinc oxide-eugenol-based endodontic sealers have been used for many years, but release

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potentially cytotoxic concentrations of eugenol (Hume 1986, Schmalz *et al.* 2000). Calcium hydroxide-based sealers promote calcification but tend to dissolve overtime and compromise the endodontic seal (Hovland & Dumsha 1985, Huang *et al.* 2002a). A new calcium hydroxide-based sealer, Acroseal (Specialités-Septodont, Saint Maur-des-fossés Cedex, France), appears to have lower solubility than other calcium hydroxide sealers, probably because of its epoxy resin component (Eldeniz *et al.* 2007). Glass–ionomer sealers may bond tooth structure but also may activate the release of prostaglandins in periapical tissues (Willershausen *et al.* 2000). Resin based sealers are increasingly gaining popularity, despite their well-documented toxicity and mutagenicity (Schweikl *et al.* 1988, Huang *et al.* 2002b). Recently, preliminary clinical data with silicon based sealers are yielding promising results (Wu *et al.* 2002, Huumonen *et al.* 2003). Research in Endodontics has increased the potential application of different biomaterials, as the biological risk of sealers is relatively high compared with other dental materials currently available.

Continuing advances in the field of implantable calcium phosphate biomaterials have produced promising results. It is well known that calcium phosphate cement (CPC) has a high biocompatibility because its composition is almost identical to that of tooth structure and bone mineral (Hong *et al.* 1990, Yuan *et al.* 2000). Its high biocompatibility makes these materials useful in applications in which cement is in contact with vital tissue (Fukase *et al.* 1992). Therefore, it continues to be a useful material for endodontic therapy (Sugawara *et al.* 1992, Yoshikawa *et al.* 1997, Geurtsen 2001).

Biological aspects of endodontic sealers are important in relation to their clinical usage. *In vitro* assays of cytotoxicity are increasingly being used for initial screening of new dental materials (Spangberg 1982). These methods are simple, inexpensive, reproducible and suitable for evaluation of basic biological properties of dental materials. The dimethyl thiazol diphenyl tetrazolium 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 aim of this current study was to investigate the cytotoxic effects and the long-term biocompatibility of three new root canal sealers on human gingival fibroblast (HGF) cells and the established mouse fibroblast cell line L929, and to compare the results with a commercial calcium hydroxide-based sealer (Acroseal).

## Materials and methods

### Preparation of cements

Three polymeric calcium phosphate cements and calcium hydroxide cement were evaluated (Table 1):

*Preparation of calcium phosphate cement powder*

The powder of calcium phosphate cements was derived from a mixture of monocalcium phosphate (MCP), calcium oxide (CaO) and synthetic hydroxyapatite (SHAP6). Both the monocalcium phosphate and the calcium oxide powder were crushed separately in an agate mortar, and then sieved to obtain an average particle size of 80 microns. A mixture of MCP and CaO was prepared at a Ca/P ratio of 1.67, the same ratio present in bone and dentine (Jarcho 1986). This mixture constitutes 60% of the total weight of the powder. A 40% weight of synthetic hydroxyapatite (SHAP6) molecules sintered at 600 °C (Abd-el & Selim 1991) were added to increase the strength of the cement powder and to act as seeds for nucleation of more hydroxyapatite (Bermudez *et al.* 1994). The powder was sieved and mechanically mixed in a shaking apparatus (Shaking apparatus Turbula; Pharmaceutical faculty, Frankfurt, Main, Germany).

*Preparation of aqueous solutions of polymeric liquids*

Three types of polymeric liquids (previously mentioned in Table 1) were each mixed individually with the calcium phosphate powder: (i) modified polyacrylic acid; (ii) the modified polyalkenoic acid of glass–ionomer; and (iii) a 35% (w/w) aqueous solution of poly methyl vinyl ether maleic acid, prepared by dissolving 35 grams of the white powder of poly methyl vinyl ether maleic anhydride (PMVE-MA) copolymer (molecular weight 50 000) in 100 mL of distilled water at 60 °C. This solution was kept at this temperature for 24 h in a shaker incubator. The 35% w/w aqueous solution of PMVE-MA was mixed with CPC powder to form the polymeric CPC cement (Type III cement).

*Preparation of calcium phosphate based–sealers*

The polymeric calcium phosphate-based cements were prepared as follows:

Type I: CPC powder: (MCPM + CaO) + SHAP6 (60% w + 40% w)

Liquid: Aqueous solution of polyacrylic acid.

Type IIa: CPC powder (MCPM + CaO) + SHAP6 (60% w + 40% w)

**Table 1** Composition of the materials used in this study and their manufacturers

| Material | Composition | Trade name | Manufacturers |
|---|---|---|---|
| Monocalcium phosphate monobasic (MCPM) | | Calcium Phosphate Monobasic | Sigma-Chemical Laboratories, St Louis, MO, USA |
| Calcium oxide (CaO) | | | Adwic Chemical Laboratories, Cairo, Egypt |
| Polyacrylic acid | Liquid: aqueous solution of polyacrylic acid and intaconic acid, tartaric acid as stabilizer (70% water, 30% polyacrylic acid) | G.C.R. | Advanced Research Inc., Dental division Leeds, England |
| Synthetic hydroxylapatite ($SHAP_6$) | Chemically precipitated using appropriate amount of $Ca(OH)_2$ and concentrated phosphoric acid to maintain Ca/P ratio of 1.67. Suitable conditions of temperature and pH as well as ageing time were maintained during precipitation of HA. HA precipitate was identified using XRD and IR spectroscopy | | Prepared at the Medical College of Georgia, Augusta, GA, USA |
| Glass–ionomer liquid modified polylalkenoic acid | Aqueous solution of polyalkenoic acid (70% water, 30% polyalkenoic acid) | Multi–Cure glass–ionomer band cement | 3M Unitek, Monrovia, CA, USA |
| Polymethyl vinyl ether maleic anhydrate copolymer (white powder) PMVE-MA | For preparation of 35% aqueous solution of polymethyl vinyl ether maleic acid | | Sigma-chemical Laboratories St Louis, USA |

Liquid: Aqueous solution of modified polyalkenoic acid.

Type III: CPC powder (MCPM + CaO) + SHAP6 (60% w + 40% w)

Liquid: A 35% (w/w) aqueous solution of poly methyl vinyl ether maleic acid.

## Cytotoxicity samples

Specimens were fabricated under aseptic conditions to avoid the need to disinfect them before cell-culture testing (Wataha *et al.* 1992). Calcium hydroxide cement was prepared according to the manufactures' instructions on a sterile glass plate. The base and catalyst were mixed in a ratio of 1 : 1.

Liquids previously mentioned were mixed with the calcium phosphate powder in a ratio of 4 : 1. This ratio was selected for the preparation of the three types of cements because as it demonstrated good handling characteristics and working time.

Six discs for each cement (Types I, IIa and III CPCs, and calcium hydroxide cement) (Acroseal) were fabricated in sterile Teflon® molds (5.5 mm in diameter and 3 mm thick) (Nationwide Plastics, Dallas, TX, USA). Each cement was packed into the mold and allowed to set at room temperature (25 °C) before testing. Immediately after setting, the specimens ($n = 6$) were removed from the molds and were used directly in cell-culture tests. Teflon discs were used as negative controls. Materials were tested for *in vitro* cytotoxicity in direct contact format (International Organization for Standardization 1993) using human gingival fibroblast (HGF) cells obtained from the Medical College of Georgia School of Dentistry clinics, and the murine fibroblast cell line L929 obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

Primary cultures of HGFs were established from healthy (non-inflamed) tissue removed during routine surgical procedures using a slight modification of a previously established protocol (Lapp *et al.* 1995). Explants were rinsed three times in Hanks buffered salt solution (HBSS) (Life Technologies, GIBCO, Rockville, MD, USA) containing penicillin G (100 U $mL^{-1}$), streptomycin (100 μg $mL^{-1}$) and Fungizone (amphotericin B, 1 μg $mL^{-1}$) (Atlanta Biological, Norcross, GA, USA). Explants were disinfected in HBSS containing 0.5% sodium hypochlorite for 5 min. After three rinses in HBSS, tissues were finely minced into pieces of about 1–2 $mm^3$. These were placed in tissue culture dishes and allowed to dry slightly. The pieces were then carefully layered with Dulbecco's modified eagle's medium (DMEM) (Life Technologies) containing non-essential amino acids, penicillin-steptomycin-fungizone (0.5 μg $mL^{-1}$) and 10% fetal bovine serum (FBS)

(Atlanta Biologicals, Lawrenceville, GA, USA). The explants were left undisturbed at 37 °C in humidified 95% air and 5% $CO_2$. After approximately 1 week, cells were visible as outgrowths from explants. At this point, the medium was changed to DMEM with 2% FBS, 8% Nu Serum V (Collaborative Research, Bedford, MA, USA) without fungizone. Fibroblasts were harvested with 0.05% trypsin, 0.53 mmol $L^{-1}$ EDTA (Sigma-Aldrich, St. Louis, MO, USA) after 2–3 weeks, pooled into a single flask, and used between passages 3 through 10.

The murine fibroblast cell line (L929) was obtained from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich, USA) supplemented with 5% fetal calf serum (Sigma-Aldrich), penicillin (125 units $mL^{-1}$), streptomycin (125 μg $mL^{-1}$) and 200 mmol $L^{-1}$ glutamine (all from GIBCO BRL, Grand Island, NY, USA).

Twenty-four hours prior to the addition of the specimens, the human gingival fibroblast cells were plated at $4 \times 10^4$ cells per well in 24 well plates (Costar, Cambridge, MA, USA) with 1 mL of medium per well. The L929 cells were plated at 25 000 cell $cm^2$ also in a 24-well format with 1.0 mL of medium per well.

### Experimental design and analysis

After setting, the specimens were secured into position with the cells such that there was 1.2 $mm^2$ $mL^{-1}$ of surface area of the specimens per millilitre of culture medium within the range of 1–2 $mm^2$ $mL^{-1}$ as recommended by the International Standards Organization (International Organization for Standardization 1992). Specimens remained in direct contact with the cells for 24 h after which the mitochondrial activity was assessed. After this interval, the specimens were removed from cell culture, rinsed twice with sterile phosphate-buffered saline (PBS), and then immediately added to a second cell culture. This second culture was incubated for 48 h before the specimens were again placed in contact with the cells. Cells with specimens were again incubated for 72 h after which the mitochondrial activity was measured. After this interval, the specimens were removed from the cell culture, rinsed twice with 18% sterile distilled water and stored in sterile PBS for 96 h. The entire set of experiments was repeated to assess the reproducibility of the assay as applied to these materials. The ageing times were selected to obtain an indication of the trend of cytotoxicity overtime. Cellular activity was assessed by measuring mitochondrial succinic dehydrogenase (SDH) activity at 24 h, 1 week, 2 week, 3 weeks and 4 weeks. This activity was measured by means of the MTT colorimetric assay (Mosmann 1983). The MTT test was done in a 1.0 mol $L^{-1}$ solution of sodium succinate at 1 mg $mL^{-1}$ MTT for 45 min. Formazan produced by the active cells was dissolved into a dimethyl-sulfoxide/NaOH solution, and then quantified by measuring colour at 562 nm. The cytotoxic effect was expressed as a percentage of the Teflon negative controls in each cell-culture plate. Differences between Teflon and each material was estimated using analysis of variance with Tukey *post hoc* tests ($\alpha = 0.05$).

### Statistical analysis

The mean absorbance of the six wells containing the same extract and their standard deviations were calculated. Original optical density values of test cultures were expressed as a percentage of optical density obtained for the control medium. The absorption value obtained with the control was considered to indicate 100% viability.

## Results

The results of the MTT assay analysis show that both the calcium phosphate-based and calcium hydroxide-based sealers induced cytotoxic effects to primary human gingival fibroblast cultures and L929 cells in culture. The sensitivity of toxicity depended on the materials tested and the cell culture system used. Figures 1 and 2 show the cytotoxic effects of these different sealers on HGF cells and L929 cells respectively. All four sealers tested clearly induced cytotoxic effects. For materials tested immediately after mixing (fresh condition), all exhibited high cytotoxicity (low SDH activity, except Type III), in both types of cultured cells. Type IIa was significantly more cytotoxic ($P < 0.05$) than the other sealers in both cultures (Figs 1 and 2). The Type I sealer was severely toxic initially but improved significantly ($P < 0.05$) over the five week evaluation period in both cultures. Type III was also was significantly ($P < 0.05$) higher than the Teflon controls at week 4 for L929 cells, and at weeks 1, 2 and 5 for HGF cells.

Calcium hydroxide-based sealer (Acroseal) demonstrated cytotoxic effects on both cultures (Figs 1 and 2). However, L929 cells were more sensitive to calcium hydroxide than HGF. In general, the rank order for both cultures demonstrated the same pattern with respect to cytotoxicity. This rank order was as follows: Type II > $Ca(OH)_2$ > Type I > Type III.

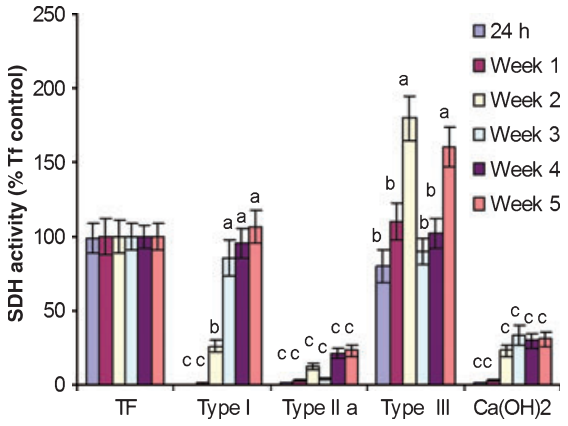


**Figure 1** Cellular response to endodontic sealers placed immediately after mixing into contact with HGF cultured cells. Cytotoxicity was evaluated at six time points initially: 1st (24 h) and after 1 week, 2 weeks, 3 weeks, 4 weeks and 5 weeks of storage in sterile phosphate-buffered saline. Succinic dehydrogenase activity was measured and expressed as a percentage of Teflon® (Tf) negative controls. Error bars indicate 1 SD of the mean ($n = 6$) .Within each set of columns, different letters indicate statistical differences (ANOVA, Tukey $\alpha = 0.05$).

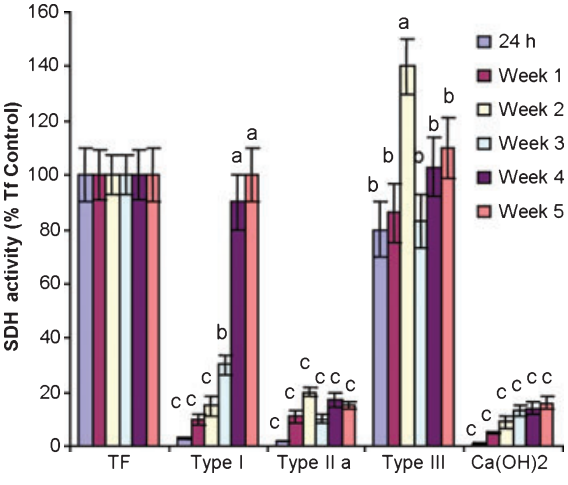


**Figure 2** Cellular response to endodontic sealers placed immediately after mixing into contact with L929 cultured cells. Cytotoxicity was evaluated at six time points initially: 1st (24 h) and after 1 week, 2 weeks, 3 weeks, 4 weeks and 5 weeks of storage in sterile phosphate-buffered saline. Succinic dehydrogenase activity was measured and expressed as a percentage of Teflon® (Tf) negative controls. Error bars indicate 1 SD of the mean ($n = 6$) .Within each set of columns, different letters indicate statistical differences (ANOVA, Tukey $\alpha = 0.05$).

## Discussion

*In vitro* experiments are routinely used for initial cytotoxicity screening of dental materials. Various cell lines (normal, diploid or transformed) are commonly used in cytotoxicity evaluations. The aim of this investigation was to evaluate the cytotoxicity of various types of root canal sealers with respect to time using one permanent murine cell line and one primary human cell line. The MTT assays are colorimetric methods for quantifying viable cell numbers. The methyl-tetrazolium ring is cleared by mitochondrial dehydrogenases in viable cells to formazan, which has a blue colour and can be measured with a spectrophotometer (Mosmann 1983). The amount of formazan produced is directly proportional to the total viable cell number over a wide range of cell numbers. The advantages of this method are its simplicity, rapidity and precision, in addition, it does not require radioisotopes. Data suggests that substantial differences in cytotoxicity exist amongst root canal sealers. However, a similar reaction was observed amongst the L929 and human oral fibroblasts for Type II calcium phosphate-based root canal sealer, the calcium hydroxide-based sealer, (both of which were highly toxic) and from the more compatible materials, Type I and Type III Calcium phosphate-based root canal sealers. This was in agreement with Schweikl & Schmalz (1996) where no differences were found between the cytotoxic reactions of L929 cells and four different cell types from oral tissues towards the material test. This finding also agrees with studies by Kettering & Torabinejad (1984), and studies by Spangberg *et al.* (1993). Both of these studies have shown similar reactivity patterns between transformed and normal diploid cells.

The biocompatibility of endodontic sealers is of particular importance as they are in direct contact with the periapical tissues. Because of this, any degradation products may elicit adverse effects. The results of the current study showed that all types of sealers tested pose significant biological risks, particularly in the freshly mixed condition. When first mixed, all the materials except Type III were severely cytotoxic. The freshly mixed condition is relevant to clinical use because sealers are placed in the canals unset and must set *in situ*. Thus, it appears that the biological risks of sealers are relatively high compared with other dental materials currently available, leaving much room for improvement.

Under the conditions of the current study, the results of cytotoxicity testing of the materials showed that

Type I CPC was initially severely cytotoxic to mitochondrial activity, but that it exhibited reduced cytotoxicity overtime. Therefore this suggests that it is possible that the initial suppression was caused by release of native mass from the materials, and that overtime, the leaching of cytotoxic components into the cell culture may be limited. Long-term evaluation of Type I calcium phosphate-based sealer has not been previously reported. Type IIa calcium phosphate-based sealer (mixed with glass–ionomer liquid), showed no improvement in cytotoxicity. Note: Type IIa differs from Type II in our other studies in that Type IIa is dually cured (chemical and light) and Type II is a light cured only version from a different manufacturer. This suggests that leaching out of components with biological liabilities was ongoing even at 4 weeks. The presence of fluoride ions may be responsible for the cytotoxicity of these materials (Doherty 1991). This leaching may have led to a greater change in the pH of the culture medium, resulting in more cellular damage and showing a reduced cell viability.

Type III calcium phosphate-based sealers mixed with an aqueous solution of poly methyl vinyl ether maliec acid, exhibited high compatibility equivalent to Teflon over the entire study period. Type III sealer was the least cytotoxic material tested, and was markedly better than the other sealers. Poly methyl vinyl ether maliec anhydride (PMVE-MA) is a commercial polymer. It can be dissolved by hydrolysis of the anhydride group in water to form the corresponding maliec copolymer. This copolymer has already a number of non-dental applications which suggest a potentially favourable biocompatibility for dental and other biomedical uses (Matsuya *et al.* 1996).

The calcium hydroxide-based sealer (Acroseal) proved to be strongly cytotoxic in both cultures. These results are in agreement with Eldeniz *et al.* (2007). The source of toxicity may be explained by the presence of amine in the epoxy base of this material that can easily diffuse into the cell-culture medium. The data obtained in this study indicate that L929 cells were generally more sensitive than HGF cells to the calcium hydroxide-based sealers.

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 sealers and reported to be more prone to toxic products than HGF (Pissiotios & Spangberg 1991). Despite the popularity of L929 cells, their use for cytotoxicity experiments is sometimes questioned. The main argument against their use is that these cells have a heteroploid chromosome pattern and may respond differently to toxic materials than the relevant target cells in humans (Kasten *et al.* 1982, Browne 1985). Furthermore, the normal diploid cells have different mitochondrial function compared with aneuploid cell lines derived from other tissue and species (Huang *et al.* 2002b) as well as more tolerance to toxic products (Al Nazhan & Spangberg 1990).

From a biological stand point, the use of human oral cells derived from the target tissues is relevant to the clinical setting. Therefore, the use of both HGF and L929 was well justified in the present study. The current study also demonstrated the importance of assessing the cytotoxicity of the sealers at multiple times. Based on the results obtained in these studies, Types I and III cements show improved biocompatible compared with the control, making them suitable for Endodontic practice. It is essential, however, to investigate further the biological and other important characteristics of these sealers both *in vitro* and *in vivo*. The results of the present *in vitro* assays may not be directly comparable with the *in vivo* conditions, where multiple healing parameters are actively functioning (Spangberg 1977). However, the results obtained from the current *in vitro* cytotoxic studies provide a positive view of the biological effects and clinical potential of these materials.

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