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The biocompatibility of modified experimental Portland cements with potential for use in dentistry

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Abstract

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Aim To evaluate the biocompatibility of a group of new potential dental materials and their eluants by assessing cell viability.

Methodology Calcium sulpho-aluminate cement (CSA), calcium fluoro-aluminate cement (CFA) and glass–ionomer cement (GIC; Ketac Molar), used as the control, were tested for biocompatibility. Using a direct test method cell viability was measured quantitatively using alamarBlue™ dye, and an indirect test method where cells were grown on material elutions and cell viability was assessed using methyltetrazolium (MTT) assay as recommended by ISO 10 993-Part 5 for *in vitro* testing. Statistical analysis was performed by analysis of variance and Tukey multi-comparison test method.

Results Elution collected from the prototype cements and the GIC cured for 1 and 7 days allowed high cell activity after 24 h cell exposure, which reduced after 48 h when compared to the nontoxic glass–ionomer control, but increased significantly after 72 h cell contact. Elutions collected after 28 days revealed reduced cell activity at all cell exposure times. Cells placed in direct contact with the prototype materials showed reduced cell activity when compared with the control.

Conclusions Cell growth was poor when seeded in direct contact with the prototype cements. GIC encouraged cell growth after 1 day of contact. The eluted species for all the cements tested exhibited adequate cell viability in the early ages with reduced cell activity at 28 days. Changes in the production of calcium hydroxide as a by-product of cement hydration affect the material biocompatibility adversely.

Keywords: accelerated cements, biocompatibility, cell culture, glass–ionomer cement, Portland cement.

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Introduction

Mineral trioxide aggregate (MTA) is used mostly in endodontics as a root-end filling material. It is composed of Portland cement and bismuth oxide (Torabinejad & White 1995). The material has been shown to be biocompatible (Koh *et al.* 1997, Moretton *et al.* 2000, Camilleri *et al.* 2004), but has limited applications in dentistry due to the long setting time and low compressive strengths compared with other materials (Torabinejad *et al.* 1995). Improvement in the properties of MTA will facilitate its use for other applications in dentistry, such as apexification procedures and pulpotomies and enable the material to withstand packing pressures when restorative materials are used following pulp capping with MTA. MTA used for pulp capping or partial pulpotomy stimulated reparative dentine formation with reports showing that MTA-capped pulps resulted in complete dentine bridge formation with no signs of inflammation (Pitt Ford *et al.* 1996, Tziafas *et al.* 2002, Andelin *et al.* 2003, Faraco & Holland 2004, Nair *et al.* 2008). The same

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results were obtained when MTA was placed over pulp stumps following pulpotomy (Holland *et al.* 2001). These properties would be ideal for dressings over vital pulps as it would preserve their integrity. Newer modified MTA-like cements namely calcium sulpho-aluminate (CSA) and calcium fluoro-aluminate (CFA) cements, which have calcium silicate as the major constituent compound (Camilleri 2008a) documented improvements in setting time and compressive strength (Camilleri 2008b), giving them the potential to replace MTA in some situations.

Calcium sulpho-aluminate cement is a mixture of Portland cement, calcium aluminate and calcium sulphate. The calcium aluminate cement reacts with the gypsum present in the Portland cement, leaving the tricalcium aluminate of Portland cement free to react with the water thus producing a flash set (Neville 1981). When these reactions take place in the presence of excess calcium sulphate, the hydrated cement created sets rapidly and has high early strength and shrinkage compensation (Evju & Hansen 2001). No calcium hydroxide is produced as a by-product of hydration (Camilleri 2008a). CFA is made up of a mixture of Portland cement and flouro-aluminate, which are mixed together during cement manufacture (Uchikawa & Tsukiyama 1973). On hydration, the Portland cement forms silicate hydrate gel and calcium hydroxide. The calcium hydroxide produced in the reaction, however, readily reacts with the CFA to produce ettringite. The early production of ettringite is responsible for the high early strength of the material compared to Portland cement (Costa & Cucitore 2000).

Glass–ionomer cements (GIC) are designed to suit a wide variety of applications. Different formulations can be used as a restorative material in nonstress-bearing areas including abrasion and erosion lesions, as a cavity liner and base and as a luting cement (Van Noort 2002). GIC used as a pulp capping agent showed excellent pulp response (Felton *et al.* 1991). The material exists as a powder and liquid and many glass–ionomers are blended with freeze-dried polyacid and tartaric acid powder, thus they harden when added to water.

The biocompatibility of GIC has been well documented. GIC are toxic when freshly prepared (Sidhu & Schmalz 2001), but this property decreases with time (Schedle *et al.* 1998). The conventional glass–ionomers exhibit good biocompatibility as they set with minimal exotherm, neutralization of any free acid is rapid and the substances leached from the set cement are generally either benign or beneficial to the tissue in which the cement is placed (Nicholson *et al.* 1991). The species leached from a GIC are dependent on the initial constituents of the cement, and their biocompatibility depends on the components released (Schuurs & van Amerongen 1993). The release of fluoride and aluminium ions early in the setting reaction combined with acidity makes the cement toxic (Lubben & Geyer 2000, Savarino *et al.*2000). When fluoride is extracted, the glass–ionomer is biocompatible (Doherty 1991). Release of aluminium phosphate causes a moderate cytotoxic reaction and the release of calcium fluoride causes a marked reaction (Sogawa 1981).

The aim of this study was to evaluate the biocompatibility of a group of fast setting materials based on Portland cement and their eluants by assessing cell metabolic function. The materials had adequate physical properties and thus have the potential to serve a wide variety of applications in dentistry, similar to the GIC.

Materials and methods

The following materials were used:

1. Calcium sulpho-aluminate cement mixed in the following proportions:

Three parts calcium aluminate (Lafarge Special Cements, Nottingham, UK)

Eight parts white Portland cement (Lafarge Asland, Valencia, Spain)

One part synthetic anhydrite (Lafarge Special Cements)

2. Calcium flouro-aluminate cement (Italcementi SPA, Bergamo, Italy)

A super-plasticizing admixture (Degussa Construction Chemicals, Manchester, UK) was added to the mixing water to increase the workability of the mix and also to reduce the amount of mixing water required by the cement. GIC Batch Number: 756 464/02 (Ketac Molar; 3M, Seefeld, Germany) was used as a control.

Cell culture

A similar experimental procedure previously described by Camilleri *et al.* (2005b) was used. Human osteosarcoma cells (HOS TE 85 ECACC No. 87070202) P68 were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), 0.02 mol L^{-1} HEPES, [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid], 2 mol L^{-1} L-glutamine, 1% penicillin or streptomycin (Life Technologies, Invitrogen Ltd, Paisley, UK) and 150 μg mL^{-1} Ascorbate (Sigma Aldrich, Gillingham, UK).

Material preparation

Discs 10 mm in diameter and 2 mm in thickness were cast from each cement type. The prototype materials were mixed with water at a water/cement ratio of 0.25 as suggested by the manufacturer. The super-plasticizing admixture was added at the end of the mixing process. The dosage was adjusted to 0.8 L per 100 kg of cement used again as suggested by the manufacturer. The glass–ionomer was mixed in a powder to liquid ratio of 3.0 : 1 proportion by weight. Two discs of test material were used for the indirect test and three discs were used for the direct test. Tissue culture plastic and 10% ethanol in DMEM were used as controls for the indirect method of testing. For the direct method Thermanox™ (TMX) cover slips 0.2 mm thick and 13 mm in diameter (NUNC brand products, Rochester, NY, USA) were used as the positive nontoxic control. Thermanox™ cover slips are made from a flexible, transparent polymer plastic which is culture treated on one side for enhanced cell attachment and growth. Polyvinyl chloride (PVC) strips 10 × 6 × 1 mm (Smiths Industries Medical Systems, Portex Ltd, Hythe, UK) were used as the negative toxic control.

Biocompatibility study

The biocompatibility of the test materials was evaluated according to ISO 10 993-Part 5 (1992) using two methods: indirect testing and direct testing. In the indirect test the cytotoxicity of the eluant was evaluated. Two replicates per material were tested by placing 10 mm discs in 10 mL of DMEM. A volume of 3 mL was removed from each test sample and replaced with fresh medium at each time interval. The test samples were maintained on a roller mixer (Luckham 4RT, Burgess Hill, UK) for the duration of the elution period.

The methyltetrazolium (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Mosmann 1983) was used to assess cell metabolic function. Seeding with HOS cells P68 (1×10^5 cells mL^{-1}) of 96-well plates was performed and these were incubated for 24 h. The medium was then removed and replaced with 100 μL of test eluants. Eight replicates per elution were used thus giving a total of 16 wells tested per material under study. Elution medium from 1, 7 and 28 days was assessed at 24, 48 and 72 h cell exposure time using the MTT assay. MTT reagent, (10 μL) was added to each well using a micropipette. The 96-well plates were then incubated at 37 °C for 3 h. The wells were then transferred to a fume cupboard, the MTT was removed using a pipette and 100 μL of dimethyl sulphoxide (DMSO) was added. The plates were then shaken for 5 min at 1000 revs min^{-1}. The plates were transferred to a plate reader set at 570 nm as test wavelength and 630 nm as reference wavelength. Standard culture medium was used as a negative nontoxic control and 10% ethanol in DMEM was used as the positive toxic control.

For the direct biocompatibility evaluation the alamarBlue™ (Serotec, Oxford, UK) test was used. Three replicates were used for each time point. The materials were cast as in the previous experiment and were cured at 37 °C and 100% humidity for 1 day after which the samples were removed from the moulds, washed with absolute alcohol for 3 min, air-dried and transferred to a sterile 24-well plate. Alcohol was used to sterilize the discs prior to seeding with cells. The materials under study were not soluble in organic solvents and were only affected by inorganic chemicals (Camilleri *et al.* 2004). TMX was used as the negative nontoxic control and PVC strips were used as the positive toxic control. The cells were seeded directly on the test materials and controls at 3.6×10^5 cells mL^{-1} and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. At selected time points 1, 7, 14, 21 and 28 days, medium was removed from the wells containing the test discs. An aliquot of 1 mL of alamarBlue™ diluted 1 : 10 in phenol red-free medium was added to each well and incubated for a further 4 h at 37 °C, 5% CO_2. Wells without any cells were used as the blank control. Following incubation, 8 × 100 μL aliquots from each well were taken and transferred to a 96-well plate for reading. Absorbance was read on a fluorescent plate reader at an emission wavelength of 590 nm (excitation wavelength 560 nm).

Statistical analysis

Data were evaluated using SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, USA). The distributions were first evaluated to determine the appropriate statistical test. The data was plotted and the distribution curve was analyzed together with the Kolmogorov–Smirnov test with $P = 0.05$. $P > 0.05$ signified a normal distribution thus parametric tests could be performed accordingly. With normally distributed data analysis of variance (ANOVA) with $P = 0.05$ was first performed to evaluate any variation between the means. In addition, once a variance was

detected between the data analysed, the Tukey test was used to perform multiple comparison tests to determine significant differences.

Results

The results for the indirect biocompatibility testing using the MTT assay performed over cement elution over a period of 28 days are shown in Fig. 1. Figure 1(a) shows the absorbance of 1 day cement elution and controls exposed to cells for 24, 48 or 72 h. Figure 1(b) shows the absorbance of elutions after 7 days and Fig. 1(c) after 28 days. The elutions collected after 1 day (Fig. 1a) revealed high absorbance indicating high cell activity after 24 h cell exposure. The cell activity was higher than that of the nontoxic control ($P < 0.001$). There was no difference between the different cements ($P < 0.05$). After 48 h of cell contact the absorbance of the nontoxic control increased more than that of the test cements ($P > 0.05$). By 72 h all three cement types had the same cell activity as the nontoxic control. Elution collected after 7 day contact with the cement (Fig. 1b) revealed higher cell activity when compared with the nontoxic control for CSA and CFA ($P > 0.05$) and a similar cell activity for GIC ($P = 0.509$) after 24 h cell exposure. After 48 h cell exposure the GIC had cell activity similar to that of the nontoxic control ($P = 0.503$) but the test cements had a reduced cell activity ($P < 0.05$). After 72 h of cell exposure the CSA had the same cell activity as the nontoxic control ($P = 0.827$) whilst CFA and GIC had a lower cell activity ($P < 0.001$). Elution collected after 28 days in contact with the cement (Fig. 1c) revealed reduced cell viability, thus exhibiting lower absorption values at all time intervals of cell contact when compared with the nontoxic control ($P < 0.001$).

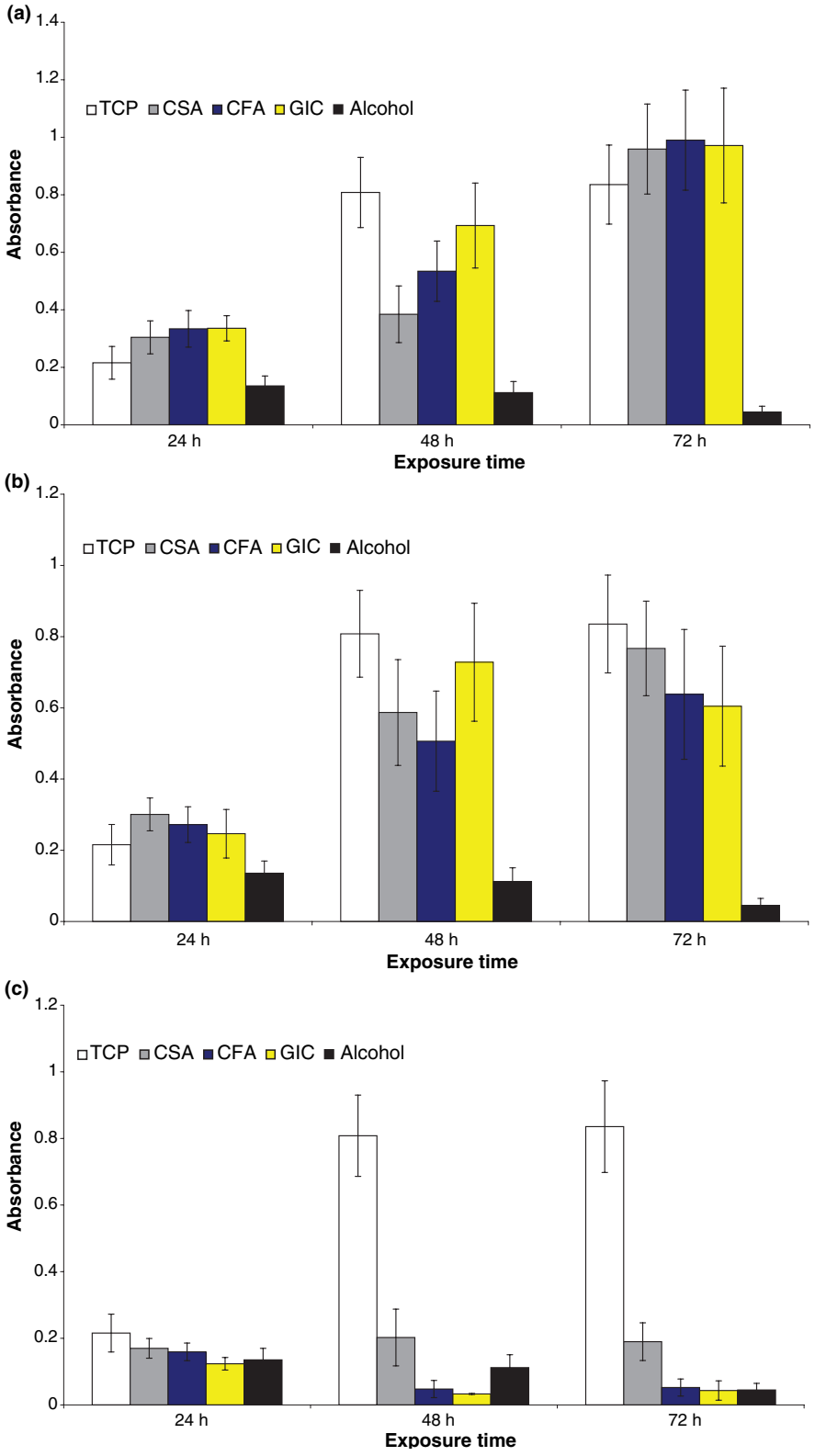
The results for biocompatibility testing using the alamarBlue™ assay are shown in Fig. 2. The prototype cements did not encourage cell growth when compared with Thermanox, the nontoxic control ($P < 0.001$). The GIC revealed a high dye absorbance up to 14 days. It had an absorbance similar to Thermanox after 1 day exposure to the cells ($P = 0.114$). The absorbance for 7 and 14 days was high but the rise was not statistically significant when compared with the nontoxic control.

Discussion

The experimental cements under study were a modification of MTA. The major constituent was tricalcium silicate, which on hydration produced a silicate hydrate and calcium hydroxide. This calcium hydroxide reacted further producing ettringite and thus it was not released as a by-product (Camilleri 2008a). The cements had a reduced setting time and higher compressive strength values than Portland cement (Camilleri 2008b). The improved physical properties could potentially make the new prototype cement useful for various applications in dentistry such as pulp capping, core build-up and as a restorative material similar to GIC.

It has been shown in a recent review that the method mostly preferred for assessment of cell growth and proliferation on MTA was scanning electron microscopy (SEM) (Camilleri & Pitt Ford 2006). Evaluation of cell growth and proliferation under SEM involves the use of gluteraldehyde in order to fix the cells, which are usually buffered with inorganic buffers such as phosphates and also the use of critical point drying that preserves the cell structure after fixing. It has been demonstrated that the use of SEM with MTA-type materials leads to the production of artefacts. The phosphate-buffered gluteraldehyde causes precipitation of phosphate crystals over the material (Camilleri *et al.* 2005a), whilst critical point drying causes the formation of calcium carbonate polymorphs over the material surface (Camilleri *et al.* 2004). The change in material surface can lead to erroneous conclusions as regards material biocompatibility. Due to the problems encountered during the experiments conducted previously, a new method for assessing biocompatibility which assessed cell metabolic function was adopted (Camilleri *et al.* 2005b). The MTT assay is dependent on the intact activity of the mitochondrial enzyme, succinate dehydrogenase, which is impaired after exposure of cells to toxic surroundings. The test involves the conversion of a tetrazolium salt, MTT, an insoluble formazan product which can be quantified by spectrophotometry. The absorbance was measured after 24, 48 and 72 h cell contact as no data were available for the prototype cements; however, in a previous publication, the

Figure 1 Material biocompatibility: mean absorbance values in MTT assay of elutions collected after (a) 1 day, (b) 7 days and (c) 28 days for the different cement types using an indirect test method with cells in contact for 24, 48 and 72 h. Tissue culture plastic (TCP) was used as the negative (nontoxic) control and 10% ethanol in DMEM was used as the positive (toxic) control. Results are ±SD showing the level of absorbance of blue formazan produced by viable cells ($n = 16$).



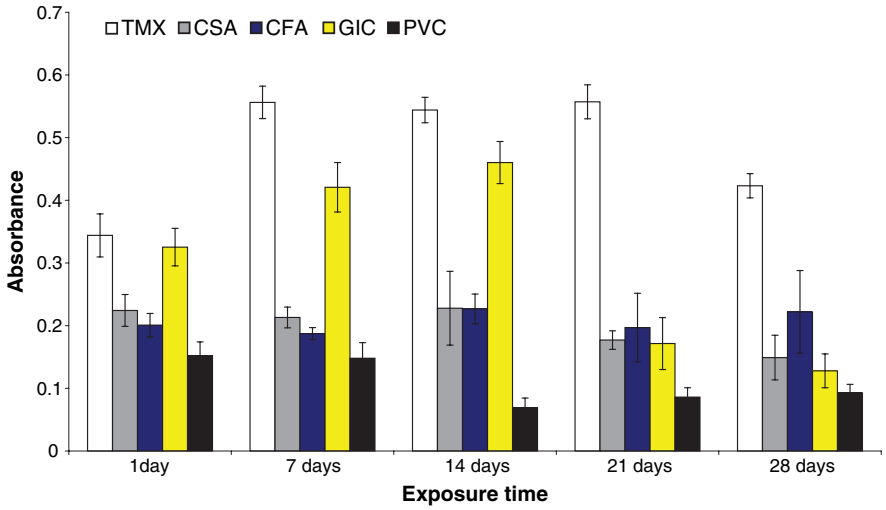


Figure 2 Material biocompatibility: mean absorbance values in alamarBlue™ direct viability assay. Cell viability was assessed over a period of 28 days. The negative nontoxic control was Thermanox™ and the positive toxic control was PVC. Results are ±SD showing level of absorbance of alamarBlue™ for each cement (n = 24).

absorbance of Ketac Molar was measured after 72 h of cell contact (de Souza Costa *et al.* 2003). Use of MTT assay on materials with a similar chemical composition as MTA also measured cell activity after 72 h of cell contact (Camilleri *et al.* 2005b). Phosphate-buffered saline was avoided in all parts of the experiment and substituted with phenol red-free medium to avoid precipitation of calcium phosphate crystals (Camilleri *et al.* 2005a). Cell viability was determined using the alamarBlue™ assay. AlamarBlue™ is a redox indicator that can be used to quantitatively measure cell viability (O' Brien *et al.* 2000). As the cells grow in culture, their metabolic activity maintains a reducing environment in the surrounding culture medium, whilst growth inhibition produces an oxidised environment. Reduction causes colour change in the alamarBlue™ indicator from nonfluorescent (blue) to fluorescent (red).

In most of the biocompatibility studies carried out on GIC very little importance has been given to the chemical reactions of the material during and after setting. This could be the reason, why inconclusive results have been obtained. The various brands release different by-products on reaction. Using the MTT and alamarBlue™ assay to study the biocompatibility of the cements enabled the material and the leachate to be assessed independently.

The GIC exhibited increasing cell activity after 1 day of direct cell contact. This is again in accordance with a previous experiment where viable cells were viewed under the scanning electron microscope after 7 day incubation on fresh GIC (Camilleri *et al.* 2008), but in contrast to another study (de Souza Costa *et al.* 2003) that found that Ketac Molar produced a reduction in cell metabolism when incubated for 72 h. The biocompatibility of the Ketac Molar was assessed using the MTT assay. Aged material was not biocompatible as shown in a previous study (Camilleri *et al.* 2008) where cells were grown on 28 day cured material with sparse cell growth observed. This is in contrast to a previous study (Schedle *et al.* 1998) which reported conventional GIC to have minimal cytotoxicity that decreased with time. Cells exposed directly to the prototype materials had reduced cell metabolism at all time intervals. This is in accordance with a previous experiment where MTA and Portland cement had reduced activity of cells in direct contact with the material (Camilleri *et al.* 2005b). In this study (Camilleri *et al.* 2005b) it was shown that it is the cement elution rather than the cement itself that was biocompatible. Both Portland cement and MTA release calcium hydroxide as a reaction by-product and this is leached out of the material at an early stage (Camilleri 2007).

Elutions collected from cements after 1 and 7 days demonstrated a high degree of biocompatibility. Both CSA and CFA release calcium hydroxide early in the hydration process (Uchikawa & Tsukiyama 1973, Evju & Hansen 2001). This release of calcium hydroxide has been shown to be responsible for the biocompatibility of

cements based on Portland cement (Camilleri *et al.* 2005b). The eluants did not encourage cell growth after 28 days. Calcium hydroxide is released early in the hydration reaction of both CSA and CFA cements. At later ages it is taken up in the reaction forming ettringite (Camilleri 2008a) and this would account for the reduced biocompatibility of 28 day elutions. Glass–ionomer also showed good biocompatibility in the elution collected after 1 day; however, the aged material seemed to be cytotoxic. The biocompatibility of GIC depends on the components released (Schuurs & van Amerongen 1993); the release of fluoride and aluminium ions early in the setting reaction combined with acidity make the cement toxic (Savarino *et al.* 2000). The reduced biocompatibility reported in GIC is caused by the release of fluoride (Doherty 1991) and aluminium phosphate (Sogawa 1981).

Conclusions

Cell growth was poor when seeded in direct contact with the prototype cements. GIC encouraged cell growth after 1 day of cell contact. The eluted species for all the cements tested exhibited adequate cell viability in the early stages with reduced cell activity at 28 days.

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