Cytotoxicity of materials used in perforation repair tested using the V79 fibroblast cell line and the granulocyte-macrophage progenitor cells

N. J. A. Souza[1], G. Z. Justo[2], C. R. Oliveira[1], M. Haun[2] & C. Bincoletto[1]

[1]Centro Interdisciplinar de Investigação Bioquímica, Universidade de Mogi das Cruzes (UMC), Mogi das Cruzes; and [2]Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, São Paulo, Brazil

Abstract

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Aim To compare the cytotoxicity of materials used to repair perforations using permanent V79 fibroblasts and murine granulocyte-macrophage progenitor cells (CFU-GM).

Methodology Set specimens from amalgam, glass–ionomer, SuperEBA, N-Rickert, MTA and gutta-percha were eluted with culture medium for 72 h and their cytotoxicities were assessed by incubating the extracts with V79 and bone marrow-derived progenitors for 24 h and 7 days, respectively. Cytotoxicity on V79 cells was judged using the total nucleic acid content (NAC), neutral red uptake (NRU) and reduction of the tetrazolium salt (MTT). The number of bone marrow CFU-GM colonies determined in clonal cultures stimulated with recombinant murine granulocyte-macrophage colony-stimulating factor was used to assess cytotoxicity to progenitor cells. Statistical analyses were conducted using the one-way analysis of variance and Tukey's test where appropriate.

Results All materials were cytotoxic in both cell systems; however, CFU-GM was more sensitive to the extracts than V79 cells. A similar rank order of toxicity was observed in V79 cells using the NAC and the MTT assays: glass–ionomer > N-Rickert ≅ SuperEBA > gutta-percha > amalgam ≅ MTA ($P < 0.05$). In contrast, the NRU test exhibited a lower sensitivity to MTA, gutta-percha and amalgam extracts. In the clonal culture assay, the toxicity was less pronounced in the presence of gutta-percha, SuperEBA and MTA. Similar cellular responses were found by placing the set specimens directly in the clonal culture dishes.

Conclusions The sensitivity of toxicity depended on the choice of the endpoint and the cell-culture system. Nevertheless, MTA was ranked as the least cytotoxic cement in both cell systems.

Keywords: biocompatibility, cytotoxicity, endodontic materials, granulocyte-macrophage progenitor cells, V79 cells.

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Introduction

Root perforation created iatrogenically during root canal treatment or as a consequence of root resorption results in communication between the pulp space and periodontal tissues (Sinai 1977). Variables that affect the prognosis of perforation repairs included: its location and size, the period of time between damage and repair, bacterial contamination, and the sealing capacity and biocompatibility of the repair material (Makkawy *et al.* 1998). In general, dental material must be biocompatible to minimize adverse effects on periodontal tissues and alveolar bone induced by direct contact. *In vitro* methods have been recommended for evaluating the cytotoxicity of endodontic materials and several

Correspondence: Prof. Claudia Bincoletto, PhD, Centro Interdisciplinar de Investigação Bioquímica (CIIB), Universidade de Mogi das Cruzes (UMC), Av. Dr Candido Xavier de Almeida Souza, 200, C.P. 411, CEP 08701-970, Mogi das Cruzes, Sao Paulo, Brazil (Tel./fax: +55 11 4798 7102; e-mail: trindade@umc.br).

model systems, using established cell lines as well as primary cells, have become available for screening purposes. In addition, implementation of distinct target cells and mechanistically different endpoint markers has been suggested for practical cytotoxicity testing of dental materials (Messer & Lucas 1999, Lovschall *et al.* 2002). This approach has been shown to be advantageous and more reliable for *in vitro* estimation of biocompatibility and toxicity mechanisms of several chemicals (Wataha *et al.* 1994, Chiba *et al.* 1998, Melo *et al.* 2003). Assays providing information about different cellular function include the reduction of the soluble tetrazolium salt (MTT), the uptake of neutral red dye (NRU) and the total nucleic acid content (NAC) (Osorio *et al.* 1998, Lovschall *et al.* 2002).

In addition to permanent cell lines such as V79 fibroblasts, predictive *in vitro* assays using murine and human progenitor cells aid considerably the estimation of toxic effects of different chemicals (Pessina *et al.* 2000). Recently, clonogenic cultures, using the bone marrow progenitor cells, have been introduced as a valuable tool in immunotoxicological studies (Parent-Massin 2001). Progenitor cells are useful not only in haematotoxicity testing, but also in the design of hybrid organs. The bone marrow contains various cell populations, including haematopoietic stem cells that give rise to progenitors of different lineages of the lympho-haematopoietic system *in vitro*, in the presence of specific growth factors. For this reason, bone marrow provides a useful cell system to study possible negative effects of biomaterials on cellular metabolism and to predict cell damage when in the presence of various chemicals (Parent-Massin 2001).

In this study, the cytotoxicity of different materials used in perforation repairs (amalgam, glass–ionomer, SuperEBA™, N-Rickert, MTA and gutta-percha) was evaluated on a permanent fibroblast cell line (V79) and on murine bone marrow granulocyte-macrophage progenitor cells (CFU-GM).

Materials and methods

Test materials and sample preparation

The materials tested were N-Rickert (F&A™, São Paulo, SP, Brazil), N-Rickert (Inodon™, São Paulo, SP, Brazil), SuperEBA™ (Bosworth Company, Skokie, IL, USA), MTA ProRoot™ (Dentsply, Tulsa, OK, USA), MTA Angelus (Angelus™, Londrina, PR, Brazil), glass–ionomer (Vitremer 3M™, Rio de Janeiro, RJ, Brazil), Amalgam GS-80™ (SDI, Bayswater, Victoria, Australia) and gutta-percha (Tanari™, Manacapuru, AM, Brazil). Three standard cylinder blocks of 10 mm diameter and 2 mm height for each tested material were prepared under aseptic conditions according to the manufacturer's directions and allowed to set for 24 h in a humidified atmosphere at 37 °C. Extraction medium was added to each sample at a 1 : 5 (wt/vol.) ratio (i.e. 1 g of test material per 5 mL of medium). Extraction was performed in an atmosphere of 5% CO_2 in air at 37 °C for 72 h. The medium was then drawn off and sterile-filtered at 0.22 μm.

V79 cell culture and cytotoxicity assays

Cytotoxicity of the extracts was assessed on a cell line derived from Chinese hamster lung fibroblasts (V79). These cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% FCS (Gibco, Grand Island, NY, USA), 100 μg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells were seeded at a density of 3×10^4 cells mL^{-1} in 96-well plates and after 48 h incubation time, the medium was replaced by dilution series of each sample prepared stepwise from 2.5 to 100% concentration. Cells were exposed for 24 h to test samples and at the end of treatment, cytotoxicity was evaluated by three independent endpoints: NAC, NRU and MTT. Three independent assays run in triplicates were performed to ensure reproducibility.

Nucleic acid content

After treatment with the undiluted and diluted extracts of test materials, cells were washed twice with cold phosphate buffered saline (PBS) and a soluble nucleotide pool was extracted with cold ethanol. The cell monolayers were then digested in 0.5 mol L^{-1} NaOH overnight at room temperature. The optical density at 260 nm of the NaOH fraction was used as an index of cell number and results were expressed as percentages of control values.

Neutral red uptake

Following removal of the undiluted and diluted extracts, cells were washed once with PBS. After 4 h incubation with serum-free medium containing 50 μg of neutral red (Sigma) per mL in a humidified 5% CO_2 atmosphere at 37 °C, cells were washed with PBS and

then 0.1 mL of a solution of 1% (v/v) acetic acid : 50% (v/v) ethanol was added to each well to extract the dye incorporated into the lysosomes. The plates were shaken for 20 min and the optical densities were read at 540 nm in a multiwell spectrophotometer (EL_X800, Bio-tek Instruments Inc, Winooski, VT, USA).

MTT reduction assay

After treatment, the undiluted and diluted extracts were removed and cells washed once with PBS. Then, 0.1 mL of a solution containing 0.5 mg 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma) per mL serum-free medium was added to each well and cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 4 h. After the incubation period, the supernatant was removed and the dark-blue formazan crystals were dissolved in 0.1 mL of ethanol. The plates were shaken for 10 min and the optical densities were read at 570 nm in a multiwell spectrophotometer.

Clonal culture of haematopoietic granulocyte-macrophage progenitors (CFU-GM) from mouse bone marrow

Assays for growth and differentiation of haematopoietic progenitors (CFU-GM) were performed using bone marrow cells collected from normal male BALB/c mice, 6–8 weeks old, bred at the University Central Animal Facilities and raised under specific pathogen-free conditions (Centro de Bioterismo, Universidade de Mogi das Cruzes, Mogi das Cruzes, SP, Brazil). Animal experiments were performed in accordance with institutional protocols of the Institutional Animal Care and Use Committee. Cell suspensions of 1×10^5 bone marrow cells were cultivated in 1 mL agar cultures in 35 mm Petri dishes. The medium used was DMEM (Sigma) containing 20% FCS (Gibco) and 0.3% agar (Difco; Becton Dickinson, Le Pont de Claix, France). Colony formation was stimulated by the addition of recombinant murine macrophage-granulocyte colony-stimulating factor (rmGM-CSF; Sigma) at a final concentration of 0.5 ng mL^{-1}. The indirect effect of test materials was assessed in the presence of dilution series of each extract prepared stepwise from 0.05 to 10% concentration. The direct effect of the materials on the CFU-GM was evaluated by placing the cylinder blocks (10 mm diameter and 2 mm height) in the centre of 35 mm Petri dishes containing the semisolid medium with progenitor cells, under sterile conditions. The cultures were incubated for 7 days in a fully humidified atmosphere of 5% CO_2 in air and colony formation (clones > 50 cells) was scored at 35× magnification using a dissection microscope. For colony typing, agar cultures were overlaid with 1 mL of 2.5% glutaraldehyde (Sigma). After 4 h, agar pellicles were floated intact onto clean glass slides, dried and stained with Luxol-Fast blue and haematoxylin. Three independent assays run in triplicates were performed to ensure reproducibility.

Statistical analysis

The results were calculated as percentage of controls and the IC_{50} (concentration that produces a 50% inhibitory effect on the evaluated parameter) were obtained from dose–response curves using the computer software package 'Origin' (version 6.0, Northampton, MA, USA). Reported results were expressed as the mean ± SD. Statistical analysis was completed by one-way analysis of variance and in cases of significant difference, the Tukey's test was used. Statistical significance was assigned when $P < 0.05$.

Results

Effect of endodontic materials on the viability of V79 fibroblasts

Extracts of all materials were cytotoxic to a permanent fibroblast cell line (V79), inducing significant alterations in cell number and viability. Using the MTT reduction test, the toxicity decreased in an order of glass–ionomer > N-Rickert (F&A^{TM}) > N-Rickert (Inodon^{TM}) ≅ SuperEBA^{TM} > gutta-percha > amalgam ≅ MTA (ProRoot^{TM}) ≅ MTA (Angelus^{TM}). As illustrated in Fig. 1, similar effects on cell viability were observed after treatment of V79 cultures with undiluted extracts of MTA (ProRoot^{TM} and Angelus^{TM}) and amalgam. In the presence of these extracts, cell proliferation was significantly impaired by 15% in comparison with control cultures ($P < 0.05$). Gutta-percha induced a slight inhibition of MTT conversion (20%) ($P < 0.05$). The effects of SuperEBA^{TM} and N-Rickert (Inodon^{TM}) extracts were comparable, decreasing cell proliferation by 30% ($P < 0.001$) of the controls. Reduction of enzyme activity due to N-Rickert (F&A^{TM}) reached 40% after 24 h exposure to the undiluted extract ($P < 0.001$). Of note, the pH values of the extracts varied between 7.0 and 7.4; therefore, no pH-induced cytotoxicity was observed.

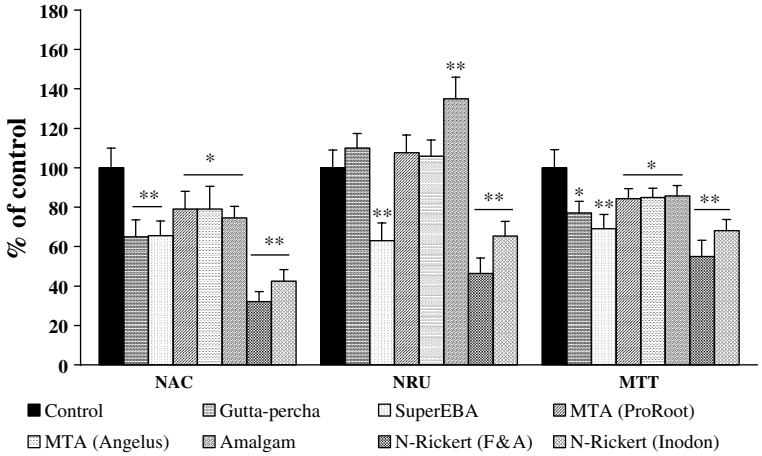

Figure 1 Viability of V79 fibroblasts after treatment with undiluted extracts of gutta-percha, SuperEBA™, MTA (ProRoot™ and Angelus™), amalgam and N-Rickert (F&A™ and Inodon™) for 24 h. Endpoints evaluated: MTT reduction (MTT), neutral red uptake (NRU) and total nucleic acid content (NAC). Results represent the mean ± SD of three independent experiments run in triplicates. *$P < 0.05$ and **$P < 0.001$ compared with control.

A similar order of toxicity was found using the NAC assay, which is based on the estimation of the cell number by the determination of their NAC. According to Fig. 1, V79 fibroblasts were least damaged after 24 h incubation with the undiluted extracts of MTA (ProRoot™ and Angelus™) and amalgam ($P < 0.05$), resulting in 20% of cell loss, followed by gutta-percha and SuperEBA™ (35%; $P < 0.001$), N-Rickert (Inodon™) (57%; $P < 0.001$) and N-Rickert (F&A™) (68%; $P < 0.001$).

Elutes of SuperEBA™ and the N-Rickert cement (Inodon™ and F&A™) also decreased the NRU ($P < 0.001$) by 37%, 35% and 54%, respectively. In contrast, MTA (ProRoot™ and Angelus™) and gutta-percha extracts did not affect significantly this endpoint compared with control. Amalgam significantly increased ($P < 0.001$) the NRU by 35% over control (Fig. 1).

Very strong dose-dependent effects on cellular functions were evident after exposure of cells to glass–ionomer extract (Fig. 2), even at low concentrations. The IC_{50} values obtained from the dose–response curves of MTT reduction and NRU were very similar (12% and 14%, respectively). However, it affected the total cell number as measured by the NAC at a lowest concentration (IC_{50} of 5%), inducing significant cell detachment and loss of cells.

Table 1 summarizes the results of the cytotoxicity study with the V79 cells, using the three endpoint assays.

Effect of endodontic materials on the growth and differentiation of CFU-GM

Similar to the findings using the permanent cell-culture system, the extracts of all materials were cytotoxic in the

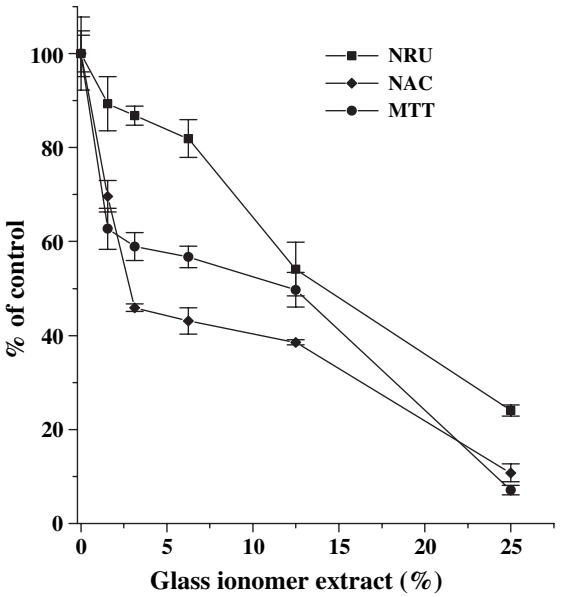

Figure 2 Viability of V79 fibroblasts after treatment with diluted extract of glass–ionomer for 24 h. Endpoints evaluated: MTT reduction (MTT), neutral red uptake (NRU) and total nucleic acid content (NAC). Results represent the mean ± SD of three independent experiments run in triplicates.

CFU-GM assay. As can be seen in Fig. 3, dose-dependent effects were observed after 7 days of incubation of bone marrow progenitor cells with concentrations ranging from 0.5 to 10% of gutta-percha, SuperEBA™, MTA (ProRoot™ and Angelus™), amalgam and glass–ionomer extracts. The decrease in colony formation was less pronounced in the presence of gutta-percha, SuperEBA™ and MTA (ProRoot™ and Angelus™) extracts (IC_{50} values >10%) than glass–ionomer and amalgam

Table 1 Cytotoxicity of the endodontic materials in V79 cells as determined by three independent endpoints[a]

Repair material	MTT (%)	NAC (%)	NRU (%)
MTA (ProRoot^{TM})	−15*	−20*	No inhibition
MTA (Angelus^{TM})	−15*	−20*	No inhibition
Amalgam	−15*	−20*	+35**
N-Rickert (F&A^{TM})	−40**	−68**	−54 **
N-Rickert (Inodon^{TM})	−30**	−57**	−35 **
SuperEBA^{TM}	−30**	−35**	−37 **
Gutta-percha	−20**	−35**	No inhibition
Glass–ionomer	Total inhibition**	Total inhibition**	Total inhibition**

The minus symbol (−) denotes inhibition relative to the controls, whereas the plus symbol (+) indicates stimulation relative to the controls. *$P < 0.05$ and **$P < 0.001$ compared with the controls. MTT, reduction of the tetrazolium salt; NAC, total nucleic acid content; NRU, neutral red uptake.

[a]V79 cells were exposed for 24 h to the undiluted extracts of set specimens and at the end of treatment, cytotoxicity was evaluated by three independent endpoints.

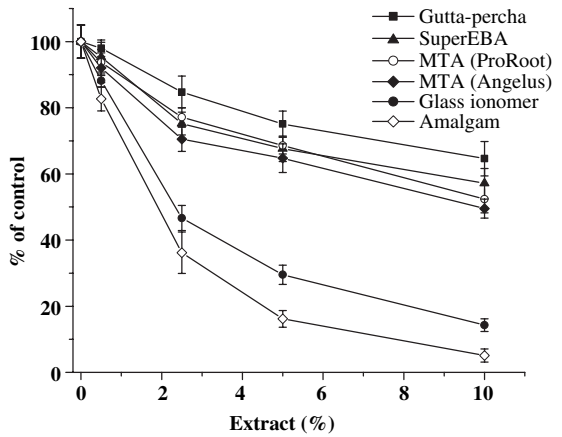

Figure 3 Effect of diluted extracts of gutta-percha, SuperEBA^{TM}, MTA (ProRoot^{TM} and Angelus^{TM}), glass–ionomer and amalgam on the growth and differentiation of mouse bone marrow granulocyte-macrophage colonies (CFU-GM) after 7 days of incubation. Results represent the mean ± SD of three independent experiments run in triplicates.

eluates, which displayed IC_{50} values of 2.3 and 2.0%, respectively ($P < 0.001$). The reaction of bone marrow progenitors to the N-Rickert cement extracts (F&A^{TM} and Inodon^{TM}) was even more dramatic, with significant inhibition of colony growth even at a concentration of 0.05%. The number of CFU-GM dropped from 106.4 ± 2.3 (control) to 3.8 ± 1.2 (N-Rickert – F&A^{TM}) and 3.0 ± 1.7 (N-Rickert – Inodon^{TM}), corresponding to approximately 96% inhibition of control CFU-GM ($P < 0.001$).

In addition to the impaired colony formation, microscopic analysis of the granulocyte-macrophage colonies after staining with Luxol-Fast blue/haematoxylin revealed that as the concentration of the amalgam extracts increased, colonies of progressively smaller size developed, whereas control cultures exhibited larger colonies of cells tightly packed, having a more condensed nucleus (Fig. 4).

All specimens analysed showed pronounced toxic effects on the growth and differentiation of progenitor cells when we placed the discs in the centre of Petri dishes containing the semisolid medium with progenitor cells. Reduced numbers of colonies were evident after cells were incubated in the presence of gutta-percha, SuperEBA^{TM} and MTA (ProRoot^{TM} and Angelus^{TM}) (Table 2), whereas amalgam, glass–ionomer and N-Rickert (F&A^{TM} and Inodon^{TM}) specimens completely inhibited colony formation.

Discussion

In the present study, V79 fibroblasts were used to study the biocompatibility of endodontic materials because of their well-defined culturing characteristics in experimental condition and their relevance as an *in vitro* system for screening purposes involving endodontic materials (Huang *et al.* 2002a, Tai *et al.* 2002, Melo *et al.* 2003).

Consistent with previous reports (Peltola *et al.* 1992, Torabinejad *et al.* 1995, Osorio *et al.* 1998, Huang *et al.* 2002b), all materials tested in this study were cytotoxic to V79 fibroblasts as determined by the multi-endpoint approach. The results revealed that MTA was the least toxic material, with no differences in the degree of cytotoxicity displayed by the trioxide aggregates from Dentsply (ProRoot^{TM}) and Angelus^{TM}. Amalgam exhibited little cytotoxicity despite the reported potential toxicity of its mercury component and tendency to release toxic elements (Kaga *et al.* 1991). Although it is considered as an inert material, gutta-percha displayed moderate levels of toxicity, followed

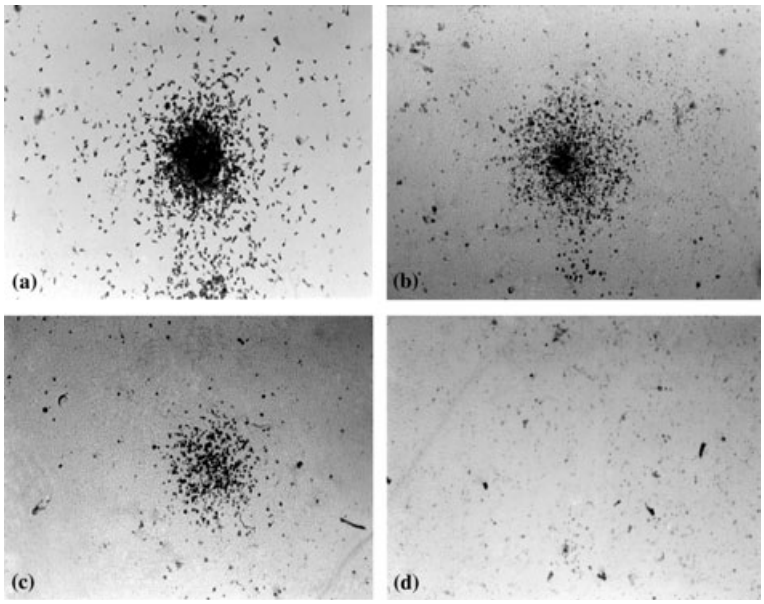

Figure 4 Representative agar cultures, showing granulocyte-macrophage colonies (CFU-GM) obtained after 7 days of incubation of mouse bone marrow progenitor cells in the absence (a) and in the presence of 2.5% (b), 5.0% (c) and 10% (d) of amalgam extract. Colonies developed in semisolid agar were fixed, air dried and stained with Luxol-Fast blue/haematoxylin. Magnification 40×.

Table 2 Direct effects of exposure for 7 days to gutta-percha, SuperEBA™ and MTA (ProRoot™ and Angelus™) on granulocyte-macrophage progenitors (CFU-GM)

Repair material	CFU-GM number	% of control	% of inhibition
Gutta-percha	7.0 ± 3.0*	6.7	93.3
SuperEBA™	5.0 ± 2.1*	4.8	95.2
MTA (ProRoot™)	4.8 ± 2.2*	4.6	95.4
MTA (Angelus™)	4.0 ± 2.1*	3.8	96.2

Results represent the mean ± SD of three experiments run in triplicates. *Indicates significant difference ($P < 0.001$) from the control value (CFU-GM number = 105.0 ± 3.4).

by SuperEBA™ (Geurtsen *et al.* 1998). Previous reports (Briseño & Willershausen 1990, Osorio *et al.* 1998) attributed their toxicity to the leakage of zinc ions into the fluids. In addition, eugenol liberated from SuperEBA™ after setting may exert a combined effect with zinc oxide (Lindqvist & Otteskog 1980). In the case of the N-Rickert cement, greater cytotoxicity was observed. Moreover, significant differences between the toxic potency of the N-Rickert from F&A™ and Inodon™ were found in all the tests, indicating a stronger effect of the material manufactured by Inodon™ on V79 cells. In agreement with previous reports (Peltola *et al.* 1992, Osorio *et al.* 1998, Huang *et al.* 2002b), glass–ionomer was the most toxic cement, exerting a dose-related inhibition of cell viability and causing detachment and loss of cells at the effective dose. Various components are segregated from glass–ionomer cements and compomers into an aqueous environment after polymerization. Co-monomers as well as additives have been implicated in the cytotoxic reactions evoked by these materials (Geurtsen *et al.* 1998). Studies have also revealed that conventional glass–ionomer cements might release fluoride into an aqueous environment (Schmalz 1994, Geurtsen 1998). Recently, fluoride was found to be a cytotoxic agent to cultured human pulp cells by inhibiting cell growth, proliferation, mitochondrial activity and protein synthesis, suggesting a contribution of fluoride release in the cytotoxicity observed for glass–ionomer (Chang & Chou 2001). This hypothesis is corroborated by the fact that in screening tests, freshly prepared ionomer is more cytotoxic than samples verified times after setting. Some researchers have also reported that some cell systems are more susceptible than others (Craig & Powers 2002).

Noteworthy, with some materials, the neutral red retention assay exhibited a lower sensitivity in comparison with the NAC and MTT methods. As determined by the NRU, gutta-percha and MTA (ProRoot™ and

Angelus™) had no effects on V79 fibroblast viability, whereas amalgam significantly stimulated the incorporation of the supravital dye. A similar discrepancy between the relative effects of trivalent chromium on cellular viability and lysosomal activity was documented by Messer & Lucas (1999). Apart from the specific lysosomal alterations induced by their interaction with metals, increase in NRU was induced by lysosomal swelling agents such as weakly basic substances and metals (Repetto & Sanz 1993, Chiba *et al.* 1998). Taken together, these results reinforce previous evidence suggesting the use of multiple, mechanistically different endpoint parameters in the toxicity evaluation of dental materials (Lovschall *et al.* 2002).

It has been well established that potentially toxic components leaching out of a variety of biomaterials used in restorative procedures and surgical endodontics may diffuse into the tooth pulp or the gingiva and thus may reach the saliva and the circulating blood. In this condition, many types of cells are sensitive to the adverse effects of the materials including the various circulating system populations, as well as the circulatory system that supports and helps maintain these cells. Macrophages are important in wound healing, and likely to be important in any inflammatory response. *In vitro* assays for initiation of inflammatory and immune reactions to dental biomaterials and their components, particularly alloys and some filling materials, have been developed to simulate and predict biological responses *in vivo* (Rakich *et al.* 1999, Heil *et al.* 2002). Macrophages react adversely to some dental material components at similar concentrations as other cell types found in the oral cavity (Wataha *et al.* 1995, Noda *et al.* 2003). As macrophages are derived from bone marrow progenitor cells, in this study, we set up direct (discs placed in the culture dishes) and indirect (extracts eluted from the set materials) tests to assess the cytotoxicity of the materials to CFU-GM.

The present results showed that all materials studied offer significant biological risks, as the CFU-GM were extremely sensitive to all biomaterials studied. Both tests, direct and indirect, indicated that gutta-percha, SuperEBA™ and MTA (ProRoot™ and Angelus™) were less toxic than amalgam, glass–ionomer and N-Rickert (F&A™ and Inodon™). Dose–response relationships were obtained for all extracts except for the N-Rickert, which showed a severe inhibition of cell growth and differentiation at a lower concentration. In relation to the fibroblast culture, CFU-GM progenitors were more sensitive to the extracts eluted from all materials, as lower concentrations were required to inhibit the development of colonies than those able to induce cell damage in the V79 system. In contrast to the findings with V79 cells, amalgam was found to be more toxic than SuperEBA™ in the clonogenic assay.

Although the cell systems used in this study differed significantly from each other, the higher sensitivity of progenitor cells may be partly explained by the fact that bone marrow undergoes rapid turnover, becoming more susceptible to toxic agents. Moreover, in the CFU-GM assay, cellular toxicity was assessed after a longer exposure time to the biomaterials. Nevertheless, these results suggest that the clonal culture of haematopoietic progenitors is an indicator of cellular damage. Therefore, its application in biocompatibility studies of endodontic materials deserves further investigation.

Conclusions

Overall, in this study, MTA cement was least toxic. The sensitivity of toxicity depended on the choice of the endpoint and the cell-culture system. Thus, multiple assays and different cell types are useful for predicting cellular damage and undesirable properties of biomaterials.

Acknowledgements

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References

Briseño BM, Willershausen B (1990) Root canal sealer cytotoxicity on human gingival fibroblasts. 1. Zinc oxide-eugenol-based sealers. *Journal of Endodontics* **16**, 383–6.

Chang YC, Chou MY (2001) Cytotoxicity of fluoride on human pulp cell cultures in vitro. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **91**, 230–4.

Chiba K, Kawakami K, Tohyama K (1998) Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. *Toxicology In Vitro* **12**, 251–8.

Craig RG, Powers JM (2002) Biocompatibility of Dental Materials. In: Powers JM, Craig R, eds. *Restorative Dental Materials*, 11th ed. St Louis, MO: Mosby, pp. 126–59.

Geurtsen W (1998) Substances released from dental resin composites and glass ionomer cements. *European Journal of Oral Sciences* **106**, 687–95.

Geurtsen W, Lehmann F, Spahl W, Leyhausen G (1998) Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *Journal of Biomedical Materials Research* **41**, 474–80.

Heil TL, Volkmann KR, Wataha JC, Lockwood PE (2002) Human peripheral blood monocytes versus THP-1 monocytes for in vitro biocompatibility testing of dental material components. *Journal of Oral Rehabilitation* **29**, 401–7.

Huang FM, Tai KW, Chou MY, Chang YC (2002a) Cytotoxicity of resin-, zinc oxide-eugenol, and calcium hydroxide-based root canal sealers on human periodontal ligament cells and permanent V79 cells. *International Endodontic Journal* **35**, 153–8.

Huang FM, Tai KW, Chou MY, Chang YC (2002b) Resinous perforation-repair materials inhibit the growth, attachment, and proliferation of human gingival fibroblasts. *Journal of Endodontics* **28**, 291–4.

Kaga M, Seale NS, Hanawa T, Ferracane JL, Waite DE, Okabe T (1991) Cytotoxicity of amalgams, alloys, and their elements and phases. *Dental Materials* **7**, 68–72.

Lindqvist L, Otteskog P (1980) Eugenol: liberation from dental materials and effect on human diploid fibroblast cells. *Scandinavian Journal of Dental Research* **88**, 552–6.

Lovschall H, Eiskjaer M, Arenholt-Bindslev D (2002) Formaldehyde cytotoxicity in three human cell types assessed in three different assays. *Toxicology In Vitro* **16**, 63–9.

Makkawy HA, Koka S, Lavin MT, Ewoldsen NO (1998) Cytotoxicity of root perforation repair materials. *Journal of Endodontics* **24**, 477–9.

Melo PS, Cavalcante HMM, Barbosa-Filho JM, Diniz MFFM, Medeiros IA, Haun M (2003) Warifteine and milonine, alkaloids isolated from *Cissampelos sympodialis Eichl*: cytotoxicity on rat hepatocyte culture and in V79 cells. *Toxicology Letters* **142**, 143–51.

Messer RL, Lucas LC (1999) Evaluations of metabolic activities as biocompatibility tools: a study of individual ions' effects on fibroblasts. *Dental Materials* **15**, 1–6.

Noda M, Wataha JC, Lockwood PE, Volkmann KR, Kaga M, Sano H (2003) Sublethal, 2-week exposures of dental material components alter TNF-alpha secretion of THP-1 monocytes. *Dental Materials* **19**, 101–5.

Osorio RM, Hefti A, Vertucci FJ, Shawley AL (1998) Cytotoxicity of endodontic materials. *Journal of Endodontics* **24**, 91–6.

Parent-Massin D (2001) Relevance of clonogenic assays in hematotoxicology. *Cell Biology and Toxicology* **17**, 87–94.

Peltola M, Salo T, Oikarinen K (1992) Toxic effects of various retrograde root filling materials on gingival fibroblasts and rat sarcoma cells. *Endodontics and Dental Traumatology* **8**, 120–4.

Pessina A, Albella B, Bueren J et al. (2000) Method development for a prevalidation study of the in vitro GM-CFU assay for predicting myelotoxicity. In: Balls M, Van Zeller M, Halder ME, eds. *Progress in the Reduction, Refinement and Replacement of Animal Experimentation*. Amsterdam, The Netherlands: Elsevier. pp. 679–92.

Rakich DR, Wataha JC, Lefebvre CA, Weller RN (1999) Effect of dentin bonding agents on the secretion of inflammatory mediators from macrophages. *Journal of Endodontics* **25**, 114–7.

Repetto G, Sanz P (1993) Neutral red uptake, cellular growth and lysosomal function: in vitro effects of 24 metals. *ATLA – Alternatives to Laboratory Animals* **21**, 501–7.

Schmalz G (1994) Use of cell cultures for toxicity testing of dental materials – advantages and limitations. *Journal of Dentistry* **22**(Suppl. 2), S6–11.

Sinai IH (1977) Endodontic perforations: their prognosis and treatment. *Journal of American Dental Association* **95**, 90–5.

Tai KW, Huang FM, Huang MS, Chang YC (2002) Assessment of the genotoxicity of resin and zinc-oxide eugenol-based root canal sealers using an in vitro mammalian test system. *Journal of Biomedical Materials Research* **59**, 73–7.

Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD (1995) Cytotoxicity of four root end filling materials. *Journal of Endodontics* **21**, 489–92.

Wataha JC, Hanks CT, Sun Z (1994) Effect of cell line on in vitro metal ion cytotoxicity. *Dental Materials* **10**, 156–61.

Wataha JC, Hanks CT, Sun Z (1995) In vitro reaction of macrophages to metal ions from dental biomaterials. *Dental Materials* **11**, 239–45.