Cytotoxicity of set polymer nanocomposite resin root-end filling materials

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Abstract

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Aim To evaluate the cytotoxicity of two forms of the novel root-end filling materials, polymer nanocomposite (PNC) resins [C-18 Amine montmorillonate (MMT) and VODAC MMT] both containing Chlorhexidine Diacetate Salt Hydrate 2%, and to compare it to that of two widely accepted commercially available materials, ProRoot® MTA and Geristore®.

Methodology Elutes of experimental materials extracted after 24 h, 1, 2 and 3 weeks were interacted with the mouse fibroblasts L-929 using a colorimetric cell viability assay (MTS) based on mitochondrial dehydrogenases activity. Using 100% and 50% concentrations of the extracted elutes of the experimental materials the effect of different concentrations of elutes on the cells was analysed. In the positive control group Hygrogold® was added to the cell culture to arrest cells bioactivity. In the negative control group, fresh Dulbecco's Eagle's minimum essential medium supplemented with 10% foetal bovine serum was used to enhance cell bioactivity. Differences in mean bioactivity values were assessed using a *t*-test and one-way ANOVA ($P < 0.05$).

Results No significant difference was found in cytotoxicity between ProRoot® MTA, Geristore® and PNC resin C-18 Amine MMT on 24 h, 1, 2 and 3 weeks samples. Sample elutes of PNC resin VODAC MMT, however, revealed cytotoxic activity during most of these experiments.

Conclusion Cytotoxicity of the elutes of PNC resin C-18 Amine MMT was not significantly different from that of ProRoot® and Geristore®. PNC resin VODAC MMT, revealed significantly more cytotoxicity compared to the other tested materials.

Keywords: cytotoxicity, nanocomposite, root-end filling materials.

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Introduction

Infected root canals harbour several species of bacteria, which can have direct and indirect effects on periradicular tissues and cause the development of periradicular pathosis. Removal of these irritants and filling of the root canal system are the main goals of nonsurgical root canal treatment. The complexity of the root canal system and the inability to completely clean it prevents successful outcomes in a proportion of cases (Torabinejad *et al.* 1993).

Although many cases with post-treatment diseases can be successfully re-treated using an orthograde approach, root-end resection followed by root-end

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filling may be the only possible treatment other than extraction, in cases where the orthograde approach is not feasible (Bergenholtz *et al.* 1979).

Because root-end filling materials come into direct contact with the periradicular tissues, their biocompatibility is considered one of the most important factors in the successful outcome of the surgical treatment. Root-end filling materials affect periradicular tissues either by direct contact or by the leached components that are released into the surrounding tissues. The outcome could be related to unexplained failures in certain cases and therefore necessitates biocompatibility testing for these material extracts as well as solid specimens (Geursten & Leyhausen 1997).

MTA is well known for its excellent biocompatibility (Keiser *et al.* 2000, Asrari & Lobner 2003) and has become the gold standard to which new root-end filling materials are being compared (Chng *et al.* 2005). It has even been shown that MTA causes upregulation of type I collagen and osteocalcin messenger RNA expression after 24 h in osteoblasts (Tani-Ishii *et al.* 2007).

Geristore® is a dual cure, hydrophilic, nonaqueous polyacid modified composite resin which has also shown favourable biocompatibility and increased cell proliferation (Al-Sabek *et al.* 2005). Studies have also indicated good attachment and spread of the cells with relatively normal morphology over the material (Al-Sa'eed *et al.* 2008).

Recently, polymer nanocomposite (PNC) resins, a new class of composite resin materials with great potential have been introduced (Giannelis 1996). PNCs are polymeric materials composed of nanoparticles such as organoclays (organically modified clays), carbon nanotubes and other nanoscale materials, which are dispersed and embedded within polymer matrix at nanoscale dimensions (Giannelis 1996). Polymer matrices with organoclays nanoparticles have exhibited greatly enhanced mechanical and thermal properties (Alexandre & Dubois 2000). In addition, these polymer nanocomposite matrices have shown improved drug elution characteristics (Cypes *et al.* 2003), which enable the material to release added medications such as antimicrobial agents like chlorhexidine during a period of time (Potluri *et al.* 2008).

Dispersion of sheet-like inorganic nanoscale silicate particles in a polymer matrix makes PNC resin filling materials superior to conventional composite resins when comparing such properties as optical clarity, strength, stiffness, thermal stability and reduced permeability (Fu & Qutubuddin 2001). In the process of polymer/clay nanocomposite synthesis, the functional-ization of clay by surfactants is considered to be a critical step (Sinha Ray & Okamoto 2003). The organic and inorganic components are typically immiscible and therefore the silicate surfaces need to be modified by attaching surfactant molecules (Messermith & Giannelis 1994). When surfactant molecules penetrate the clay layers, the montmorillonite (MMT) clay which is originally hydrophilic becomes organophilic and is then able to exfoliate (disperse) within the polymer matrix (Lan & Pinnavaia 1994). A key factor governing the degree of exfoliation of the clay layers is the extent of interaction between the surfactant molecules of the functionalized clay and the base polymer matrix (Park & Jana 2003). Generally, a high degree of interaction will result in well exfoliated clay platelets in the polymer matrix and lead to a substantial improvement in key properties of the PNC (Fu & Qutubuddin 2001).

The purpose of this study was to investigate the cytotoxicity of PNC resin elutes on permanent cell lines *ex vivo* and to compare it to that of the two root-end filling materials, ProRoot® (MTA) and Geristore®.

It was hypothesized that the cytotoxicity of the PNC was the same as that of these two widely accepted materials.

Materials and methods

Cell line and culture conditions

L-929 mouse fibroblast cells (ATCC CCL-1) were grown as monolayer culture in 100-mm culture plates (Corning; Medfield, PA, USA) using Dulbecco's Eagle's minimum essential medium (DMEM) (Cellgro®; Mediatech, Inc., Manassas, VA, USA) containing 2 mM of L-glutamine and supplemented by 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) containing 10 000 units of penicillin-G mL^{-1} and 10 mg of streptomycin mL^{-1} (Sigma Chemical Co., St Louis, MO, USA) in an atmosphere of 5% CO_2/95% air at 37 °C (NUAIRE™ IR Autoflow; CO_2 Water-Jacketed Incubator, Plymouth, MN, USA).

When the cells became confluent as a monolayer, verified as seen under light microscope (Olympus CK30; Olympus Optical Co. Ltd, Tokyo, Japan), they were seeded on 96 wells plates for studying the effect of material elutes or re-grown for the control groups. Adherent cells at a logarithmic growth phase were detached by the addition of 2 mL of a 0.05% trypsin (Gibco, 1 : 250, Invitrogen, Carlsbad, CA, USA) and 0.02% EDTA mixture and incubated for 3 min at 37°C. Then 2 mL of DMEM supplemented with FBS was

added to stop the effect of trypsin. About 15 μL of the well-mixed cell suspension was pulled for cell counting using hemacytometer (Bright-Line Hemacytometer; Hausser Scientific, Horsham, PA, USA) and the desired cell concentrations for the experiments were calculated and prepared accordingly.

Test materials and sample preparation

The experimental root-end filling materials used were tooth-coloured MTA (ProRoot®, Dentsply, Tulsa, OK, USA), Geristore® Syringeable (DenMat, Santa Maria, CA, USA) and PNC resins [nonpolymerizable surfactant C-18 Amine MMT and polymerizable surfactant VODAC (Vinylbenzyl Octadecyl Dimethyl Ammonium Chloride) MMT, both containing 2% chlorhexidine (CHX)]. Their components are summarized in Table 1. Commercial materials (ProRoot® and Geristore®) were mixed according to the manufacturer's instructions under aseptic conditions (Steril Gard Hood; The Baker Company Inc., Sanford, ME, USA) to limit the risk of microbial contamination. Ingredients of PNC resins (C-18 Amine MMT and VODAC MMT) were measured using METTLER TOLEDO (Mettler-Toledo International Inc., Greifensee, Zurich, Switzerland) and mixed inside a light-sealed container under aseptic conditions. A Coltulux light-curing system (Colten/Whaldent Inc,

Table 1 Ingredients of polymer nanocomposite resins (C-18 Amine MMT and VODAC MMT)

Initiators
  Benzoyl Peroxide, Reagent grade 97% (Sigma-Aldrich, St Louis, MO, USA)
  Camphorequinone 97% (Sigma)
  Ethyl4-(dimethyl Benzoate) 98% (Sigma)
  2-(Dimethylamino) ethyl methacrylate 98% (Sigma)
  Diphenyliodonium chloride 98% (Sigma)
Monomers
  Bisphenol A glycerolate (1-glycerol/phenoldimetharylate) (Sigma)
  Triethylen Glycoldimethacrylte 95% (Sigma)
  2-hydroxy ethyl methacrylate 97% (Sigma)
Polymer
  MetaBond L-Powder, (Parkill, Edgewood, NY, USA)
Antibacterial
  Chlorhexidine Diacetate Salt Hydrate (Sigma)
Nanoparticles
  Nonpolymerizable surfactant C-18 amine MMT (Mineral Colloid BP, Southern Clay Products, Austin, TX, USA)
  Polymerizable surfactant Vinylbenzyl Octadecyl Dimethyl Ammonium Chloride MMT
  (Synthesized in the CWRU Department of Chemical Engineering)

Mahwah, NJ, USA) with a light intensity of 600 mW cm^2 was used to cure the Geristore® and PNCs. MTA specimens were incubated in an atmosphere of 5% CO_2/95% air at 37 °C for 4 h until their initial set.

Cytotoxicity evaluation

Four experimental root-end filling materials (ProRoot®, Geristore® Syringeable, C-18 Amine MMT and VODAC MMT polymer nanocomposite resins) were moulded in prefabricated curing rings (5 mm diameter, 2 mm height). The rings were placed over sterilized glass plates to receive the materials and 6 pellets for each material were made. After the materials set, the pellets were placed under UV light for 1 h. Then each specimen was placed in a separate well of a 24-well culture plate (Costar®, Corning Inc., Corning, NY, USA) and 1 mL of DMEM was added to each well. Plates were incubated in an atmosphere of 5% CO_2/95% air at 37 °C.

Elutes at 24 h, 1, 2 and 3 weeks were tested for cytotoxicity. Elutes in DMEM media were sterilized by passing through 0.22 μm Millipore PES Membrane filters (MILLEX® GP Filter Unit, Corrighwahill, Cork, Ireland).

Cell cultures were prepared by seeding 10 000 cells/ 100 μL DMEM (supplemented with FBS) in a 96-well culture plate and incubated for 24 h in an atmosphere of 5% CO_2/95% air at 37 °C (12 wells for each experimental materials, 6 well for positive control and 6 wells for negative control groups). After 24 h, the media removed and 100 μL of filtered experimental material elutes [in 6 wells full concentration elutes (100%) and in 6 wells 1 : 1 elutes and media (50%)] were added to the wells and incubated for another 24 h at the same atmospheric condition.

Viability of the cells was assessed based on the cells mitochondrial dehydrogenases activity using a tetrazolium compound assay. In this study, CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega Corporation, Madison, WI, USA) was used which is a colorimetric method for determining the number of viable and active cells. The MTS tetrazolium compound is bioreduced by cells into a dark-blue coloured product named Formazan which is soluble in tissue culture medium (Barltrop *et al.* 1991).

MTS reagent thawed in water bath (ISOTEMP 220; Fisher Scientific, Scientific Support, Hayward, CA, USA) at 37 °C for 10 min to reach the room temperature from its −20 °C storage temperature. About 20 μL of

MTS reagent were added to the cell cultures and incubated for 4 h in an atmosphere of 5% CO_2/95% air at 37 °C. Optical density of these coloured formazan products were measured using Microplate Spectrophotometer (PowerWave TM XS; BioTek Instruments, Inc., Winooski, VT, USA) after 4 h on 490 nm wave length. Positive control group consisted of six cell cultured wells in which the media was replaced with 80 μL of DMEM plus 20 μL of Hygrogold®. In negative control group (six cell cultured wells) the media was replaced with fresh complete DMEM (containing FBS). Each individual treatment was repeated thrice to ensure reproducibility.

Statistical analysis

Data were statistically analysed using unpaired *t*-test to compare the mean values of each experimental group to those of control groups and to compare mean values of groups to other groups in a one by one basis with the null hypothesis that the means of the two groups undergoing *t*-test analysis were statistically similar. One-way ANOVA was used to compare the mean values of all experimental groups to each other; *P* values of ≤0.05 were considered statistically significant.

Results

The mean values and standard deviations of the eight experimental groups and two control groups eluted for 24 h, 1, 2 and 3 weeks can be found on Tables 2–5.

In the 24-h period, cells in all experimental groups except the pure elute (100%) of VODAC MMT were viable and was significantly different from the positive control group ($P < 0.0015$). Compared to the untreated cell cultures (negative control group), elutes of Geristore® 50% and VODAC MMT 100% reduced cell viability to 76% and 67%, respectively (Table 2). Comparing experimental groups to each other, the ANOVA revealed that pure (100%) elutes of MTA, Geristore® and C-18 Amine MMT were not significantly different from each other.

Mean values of elutes of the 1-week period time (Table 3) revealed that in all experimental groups except VODAC MMT 100%, cells had preserved their viability when compared to the positive control group, but when mean values were compared to untreated cells in the negative control group, only pure (100%) elutes of MTA and C-18 Amine MMT had similar viability to untreated cells. The pure elute of Geristore® had a mean value of approximately 60% of that for MTA and C-18

Table 2 Mean and standard deviation (SD) of MTS assay values and *t*-test results comparing the specimens to positive and negative controls in 24-h specimens

	Mean, SD	*t*-test compared to negative control, *P*	*t*-test compared to positive control, *P*
MTA (100%)	1.3720, 0.2946	0.11	<0.0015
MTA (50%)	1.3537, 0.2853	0.84	<0.0015
Geristore (100%)	1.5523, 0.1485	0.46	<0.0001
Geristore (50%)	1.2427, 0.1462	<0.0045	<0.0001
C-18 Amine (100%)	1.5388, 0.2056	0.45	<0.0001
C-18 Amine (50%)	1.6108, 0.1116	0.81	<0.0001
VODAC (100%)	1.0982, 0.3679	<0.012	0.1022
VODAC (50%)	1.5868, 0.1319	0.65	<0.0001
Positive control	0.8200, 0.0898		
Negative control	1.6353, 0.2190		

Table 3 Mean and standard deviation (SD) of MTS assay values and *t*-test results comparing the specimens to positive and negative controls in 1-week specimens

	Mean, SD	*t*-test compared to negative control, *P*	*t*-test compared to positive control, *P*
MTA (100%)	1.2653, 0.1671	0.4780	<0.0015
MTA (50%)	0.9470, 0.2565	0.031	<0.0015
Geristore (100%)	0.7687, 0.2509	0.0016	<0.0001
Geristore (50%)	0.8727, 0.3652	0.046	<0.0001
C-18 Amine (100%)	1.1233, 0.4304	0.6198	<0.0001
C-18 Amine (50%)	0.7383, 0.3799	0.012	<0.0001
VODAC (100%)	0.8507, 0.3413	0.0271	0.1022
VODAC (50%)	0.9788, 0.2240	0.03	<0.0001
Positive control	0.6455, 0.0420		
Negative control	1.1237, 0.0396		

	Mean, SD	*t*-test compared to negative control, *P*	*t*-test compared to positive control, *P*
MTA (100%)	0.7932, 0.2606	0.0027	<0.2006
MTA (50%)	1.1612, 0.2376	0.5690	<0.0002
Geristore (100%)	0.8547, 0.2944	0.0132	<0.1157
Geristore (50%)	1.2330, 0.2634	0.9000	<0.0002
C-18 Amine (100%)	0.8547, 0.1696	0.0004	<0.0150
C-18 Amine (50%)	1.0833, 0.1806	0.1018	<0.0001
VODAC (100%)	0.8972, 0.2421	0.0092	<0.0310
VODAC (50%)	0.9528, 0.2436	0.0245	<0.0049
Positive control	0.6455, 0.0295		
Negative control	1.2190, 0.0376		

Table 4 Mean and standard deviation (SD) of MTS assay values and *t*-test results comparing the specimens to positive and negative controls in 2-week specimens

	Mean, SD	*t*-test compared to negative control, *P*	*t*-test compared to positive control, *P*
MTA (100%)	1.7067, 0.2675	0.7295	<0.0001
MTA (50%)	1.4868, 0.2805	0.6598	<0.0001
Geristore (100%)	1.0455, 0.2227	0.0577	<0.0190
Geristore (50%)	1.2525, 0.3817	0.2488	<0.0131
C-18 Amine (100%)	1.5045, 0.2431	0.6992	<0.0001
C-18 Amine (50%)	1.4592, 0.1165	0.5614	<0.0001
VODAC (100%)	1.0122, 0.2182	0.0461	<0.0314
VODAC (50%)	1.4228, 0.3223	0.5182	<0.0008
Positive control	0.7680, 0.0982		
Negative control	1.6105, 0.6059		

Table 5 Mean and standard deviation (SD) of MTS assay values and *t*-test results comparing the specimens to positive and negative controls in 3-week specimens

Amine MMT. There was no significant difference when 50% elutes were compared to each other.

As can be seen in Table 4, elutes for a time period for 2 weeks of MTA 100% and Geristore® 100%, had lowered the cell viability to the extent that their mean values were not significantly different from that of the positive control group ($P < 0.2006$ and $P < 0.1157$, respectively). The mean values of MTA 50%, Geristore® 50% and C-18 Amine MMT 50% revealed that cell viability was not affected.

Mean values of elutes for a time period of 3 weeks revealed that cells were bioactive and significantly different from positive control group in all experimental groups. However, pure elutes of VODAC MMT displayed reduced cell viability to approximately 63% compare to the cells in negative control group (Table 5). When comparing the experimental groups of the elutes with the 3 weeks time period to each other, pure elutes of VODAC MMT and Geristore® reduced cell viability. However, there was no statistical difference between elutes at 50% concentration.

Discussion

Generally, the toxicity of a root canal sealer or root-end filling material is assessed using a three-step approach.

The first step is to screen a candidate material using a series of laboratory cytotoxicity assays. Then, if the material is determined not to be cytotoxic *ex vivo*, it can be implanted in subcutaneous tissue or muscle and the local tissue reaction evaluated. Finally, the *in vivo* reaction of the target tissue versus the test material should be evaluated in human subjects or animals (Osorio *et al.* 1998).

A variety of test systems are available to determine the cytotoxicity of dental materials in cultured mammalian cell populations (Schweikl & Schmalz 1996). Permeability assays monitor the integrity of cell membranes by the inclusion or exclusion of vital dyes, or by the release of radio-labelled chromium. Replication assays indirectly assess the ability of cells to proliferate by measuring the incorporation of nucleotide analogues that have been radio-labelled or are detectable by immunoassay during DNA synthesis. Changes in the cellular cytoskeleton or at the cell surface are observed by morphological studies. Functional assays typically evaluate the ability of cells to provide the energy necessary for anabolic activities, or the end-products of such activities (Keiser *et al.* 2000).

The functional assay in the present study used the tetrazolium salt MTS to assess mitochondria1 dehydrogenase activity (Mosmann 1983). It is a pale yellow

substrate that produces a dark blue formazan product when cleaved by mitochondrial enzyme activities, and so the reaction only occurs in living, metabolically active cells. Because the MTS formazan product is soluble in tissue culture medium, the CellTiter 96® AQueous One Solution Cell Proliferation Assay requires fewer steps than procedures that use tetrazolium compounds such as MTT (Bernabei *et al.* 1989). The formazan product of MTT reduction is a crystalline precipitate that requires an additional step in the procedure to dissolve the crystals before absorbance readings recorded at 570 nm (Mosmann 1983).

In the present study, specimens were prepared in the form of disc pellets (5 mm diameter, 2 mm height), the form that has been used in some other cytotoxic studies (Leyhausen *et al.* 1998, Al-Sa'eed *et al.* 2008). These pellets had a surface area/media ratio of approximately 70 mm^2 mL^{-1}, which were in accordance to ISO standard 10993-5:4.2.3.5 (International Standard Organization 1992).

In this study, elutes of the test materials was prepared for up to 3 weeks. This is the approximate time that nanocomposite resins reveal chlorhexidine elution (Potluri *et al.* 2008, Potluri 2009). Elutes can easily be sterilized by filtration, and it is possible to examine the effect of materials on cells that are both distant to and in contact with them. The use of elutes simulates the immediate post-surgical root-end environment in which toxic elements of the root-end filling material leach into the surrounding fluids in the bony crypt. Elutes can also be made in a series of concentrations to observe a possible dose–response relationship (Keiser *et al.* 2000).

Cytotoxicity assays suggested no significant differences amongst root-end filling materials except for VODAC MMT, probably due to the presence of vinylbenzyl surfactant used in the synthesis of this PNC resin material. The exact mechanism for the increased cytotoxicity should be clarified through further chemical investigations.

In the present study, every test and control group consisted of six replicates. Nelson *et al.* (1999) showed that six replicates would provide sufficient statistical power to detect changes in toxicity of <10% given the magnitude of standard deviations when $\alpha = 0.05$.

Findings of the cytotoxicity effects of MTA and Geristore® are in accordance with many other studies (Torabinejad *et al.* 1995, Osorio *et al.* 1998, Camp *et al.* 2003, Al-Sabek *et al.* 2005, Ribeiro *et al.* 2006, da Silva *et al.* 2006) that proved the biocompatibility of these commercially available materials. Results on

C-18 Amine MMT are in agreement with the findings of some of the cytotoxicity studies on composite resins, which have showed high clinical success and favourable healing (Rud *et al.* 2001) and increased proliferation of cells (Al-Sa'eed *et al.* 2008), but in contrast to others that reported significant reductions in cellular viability (Haglund *et al.* 2003) and cytotoxic activity of resin-based root-end filling materials (Tai & Chang 2000, Huang *et al.* 2002). Resin monomers in composite resins have cytotoxic effects at higher concentrations (Geurtsen *et al.* 1998, Al-Hiyasat *et al.* 2005), but may be capable of tumour initiation at relatively low concentrations (Kleinsasser *et al.* 2006).

The present study represents the screening step of toxicity evaluation of these novel root-end filling materials. Cytotoxic evaluation results of C-18 Amine are promising and show that adding nanoparticles and chlorhexidine to composite resins does not have negative effect on their biocompatibility. To have a better understanding of the intracellular events that affect viability of the cells after exposure to these elutes, exploring deeper layers of the functions of cells through evaluating different cytokines activities is suggested.

Conclusion

Cytotoxicity of the elutes of set PNCs C-18 Amine MMT and VODAC MMT resin containing 2% chlorhexidine on mouse fibroblasts was evaluated using colorimetric MTS assay for up to 3 weeks. The cytotoxicity of C-18 Amine was not significantly different from that of ProRoot® MTA and Geristore®. The other PNC resin, VODAC MMT, revealed much higher cytotoxic activity compared to the other tested materials. Further investigation through other cell functions such as cytokine production in response to these materials is suggested.

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