Research Article

Cytotoxicity and metal content of tricalcium silicate-based endodontic cements

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Abstract

Aim: To compare the cytotoxicity of six silicate endodontic cements and evaluate the concentrations of eleven metals (Ni, Cr, Fe, Pb, Cd, Zn, Cu, Al, As, Mn, Bi) in the materials.

Methods: Extracts derived from MTA CAPS, MTA Angelus White, Cerkamed MTA+, Masterdent MTA, Biodentine and ProRoot MTA White were incubated with MRC5 cells for 24 and 72 hours. The cytotoxicity was assessed by sulforhodamine-B assay. Samples of the same materials were also prepared and analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES). The statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey multiple comparison tests.

Results: MTA Angelus White displayed its higher antiproliferative effect after 24 hours (p<0.05). At 72 hours all the cements had similar effects on cell viability (p>0.05). Ni, Cr, Cd, Cu and Mn were not detected in any of the materials tested. Pd, As and Zn were found only in MTA-CAPS.

Conclusions: All the materials tested were proved highly biocompatible, except for MTA Angelus White, which showed mild cytotoxicity. Regarding the metal content the cements showed presence of some metals, although the detected concentrations were below the safety limits.

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Introduction

Mineral trioxide aggregate (MTA) has been used in numerous clinical procedures including root perforation repair [1, 2], one-visit apexification[3, 4], retrograde filling[5], pulp capping [6] and pulpotomy[7]. Several studies have demonstrated that MTA has good sealing ability[8-10] and marginal adaptation[11], excellent biological properties[12, 13] and the potential to promote hard-tissue formation and odontoblastic differentiation[14, 15]. However, there are some drawbacks accompanying the use of MTA including long setting time and difficult manipulation [16].

The first mineral trioxide aggregate formulation was a mixture of Portland cement and bismuth oxide in a ratio of 4:1. Several new calcium-silicate materials have been subsequently introduced aiming to alleviate the drawbacks of MTA, with minor variations in composition such as the inclusion of calcium chloride, calcium carbonate, silicon dioxide, or the omission of calcium sulfate[17-19].

Given the clinical applications of calcium-silicate cements and the fact that they are placed in direct contact with pulpal or periradicular

tissues, it is essential for them to be non-toxic and biocompatible. There have been several in vitro and in vivo studies evaluating the biocompatibility of MTAs and alternative tricalcium silicates. Nevertheless, studies comparing the biocompatibility of different MTA brands and new calcium-silicate cements remain sparse.

The fact that mineral trioxide aggregate is refined from Portland cement leads to suspicion of possible contaminants or trace metals constituents in the commercial products. To ensure lack of contamination, MTA manufacturers claim to use materials obtained from refined laboratory-grade materials[20]. However, previous researchers have detected traces of arsenic, lead and chromium in MTA cements[21-26].

The aim of this study was to assess the cytotoxicity of six calcium-silicate endodontic cements currently available on the market and to evaluate the concentrations of eleven heavy metals in the same materials using inductively coupled plasma-atomic emission spectrometry (ICP-AES). The null hypotheses were that the tested materials had no differences in terms of cytotoxicity and in the heavy metal content.

Materials and methods:

Cytotoxicity
Cell culture

MRC5 cells (human lung fibroblasts) supplied by the American Type Culture Collection (ATCC) were used to evaluate the biocompatibility of the calcium-silicate cements. The cells were grown in T-75 culture flasks (Costar/Corning, NY, USA) and sub-cultured twice a week at 37 ºC in an atmosphere containing 5% CO_2 in air and 100% relative humidity. The culture medium was Dulbecco's Modified Eagle Medium (DMEM, Life, NY, USA) supplemented with 10% FBS (Fetal Bovine Serum, Gibco, Glaskow, UK) and antibiotics (100IU/mL penicillin and 100mg/mL streptomycin).

Cell Inoculation

Adherent cells at a logarithmic growth phase (eight to ninth passage) were detached by the addition of 2-3 ml of a 0.05% trypsin (Gibco, 1:250) and 0.02% EDTA mixture and incubated for 2-5 minutes at 37ºC. The cells were then plated in 96-well flat-bottom microtiter plates (Costar/Corning, NY, USA) at a density of 3,000 cells per well (growth area 0.32 cm2) in 100 µL culture medium, and were left for 24 h in the incubator to resume exponential growth.

Preparation of Cement Extracts

The composition and the manufacturers of the MTA-cements examined are shown in Table 1.

Materials	LOT number	Composition	
		Powder	Liquid
MTA CAPS Acteon, Merignac, France	7405802	Mixing of mineral oxides based on calcium and tungstate	Water
MTA Angelus White Angelus, Londrina, parana, Brazil	32317	Tricalcium silicate Dicalcium silicate Tricalcium aluminate Tetracalciumaluminoferrite- Bismuth oxide	Water
Cerkamed MTA+, StalowaWola, Polska	1909141	Calcium hydroxide Silicon Iron Aluminium Sodium Potassium Bismuth Magnesium oxides Calcium phosphate	
MASTERDENT MTADentonics, Inc., Monroe, North Carolina, USA	4020018	Tricalcium silicate Dicalcium silicate	
Biodentine Septodont, Saint Maur des Faussés, France	B10981	Tricalcium silicate Dicalcium silicate Calcium Carbonate Calcium Oxide Iron oxide Zirconium oxide	Calcium chloride Hydrosoluble polymer
ProRoot MTA White Maillefer, Dentsply, Switzerland	13082005	Portland cement Tricalcium silicate Bismuth oxide Dicalcium silicate Tricalciumaluminoferrite Tetracalciumaluminoferrite Calcium sulfate dehydrate or gypsum	

Table 1: Composition and manufactures of cements tested.

The materials were prepared according to the manufacturers' instructions under aseptic conditions and placed in cylindrical teflon tubes (15 mm in diameter and 3 mm in height). The samples were allowed to set at 37ºC in 100% relative humidity for 24 hours. After setting, the pellets were weighed to the nearest of 0.001 g and exposed to ultraviolet light for 20 minutes to ensure sterility and transferred into vials containing 5 mL DMEM. The weight to volume ratio used for extract preparation was about 0.2 gr/ml. Five milliliters of extract were drawn from each well after incubation at 37 ºC in 100% relative humidity for 24 hours and filtered using a 0.22-µm syringe filter. A volume of 100 µL of extract was added to the cells (thus the final volume in each well was 200 µL) and incubated for either 24 or 72 hours. Cells treated with 100 µL of DMEM were used as negative controls. Three replicate wells were prepared for each cement and the experiments were carried out at least twice. At the end of the incubation period, cell numbers were estimated by means of the sulforhodamine-B (SRB) assay.

Sulforhodamine-B (SRB) colorimetric assay

The SRB assay was performed as described by Skenan et al[27] and modified by Papazisis et al[28]. Briefly, the culture medium was aspirated prior to fixation and 75 µL of 10% cold trichloroacetic acid were gently added to the wells. The plates were left for 30 minutes at 4 ˚C, washed five times with deionized water and left to dry at room temperature for at least 24 hours. Subsequently, 70 µL 0.4% (w/v) sulforhodamine B (Sigma Aldrich Corp., St. Louis, MO, USA) in 1% acetic acid solution were added to each well and left at room temperature for 20 minutes. The SRB was removed and the plates were washed five times with 1% acetic acid before air-drying. Bound SRB was solubilized with 70 µL 10 Mm unbuffered Tris-base solution (E.Merck, Darmstadt, Germany) and the plates were left on a plate shaker for at least 10 minutes. Absorbance was read at 492 nm by subtracting the background measurement of 620 nm. The test optical density (OD) was defined as the mean absorbance of each individual well minus the blank value ('blank' is the mean OD of the background control wells). Mean values and coefficient of variation (CV) were calculated. Each experiment was carried out at least twice. The results were expressed as a 'survival fraction', which was calculated as the percentage of test OD to control OD (plain medium was added to the control wells).

Heavy metal analysis

Sample preparation

Four samples were prepared for each material (n=4). Each sample (0.2 g of MTA in unhydrated powder form weighed to the nearest of 0.001 g)was transferred to a 100 mL Teflon tube (Sanplatec, Osaka, Japan). A mixture of 2 mL HNO_3(HNO_3 65%, Chem-Lab, Zedelgem, Belgium), 3 mL HCL(HCl 37%, Carlo Erba Reagents, Italy), and 2 mL HF(HF 48%, Merck Millipore, Massachusetts, USA) was added to the Teflon tube and left to stand for 1hour. The tube was capped and heated to 120°C on a heating block and then allowed to equilibrate for 2hours. Special precautions were taken when handling the hydrofluoric acid and the digestions were made in a ventilated hood. The temperature of the mixture was increased gradually to avoid the loss of metal traces by abrupt boiling. After the reaction, the mixture was cooled to room temperature, filtered through Whatman No. 40 filter paper (WhatmanPlc, Maidstone, UK) and the clear digests were quantitatively transferred to 25 mL volumetric flasks and diluted to final volumes with de-ionized water. A blank test was performed in parallel using the same procedure and the same quantities of all reagents except for the test sample.

Instrumentation

The resulting filtrates were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES). All ICP-AES measurements were carried out using a Perkin Elmer (MA, USA) Optima 3100XL axial viewing spectrometer according to the operating condition described in Table 2. The injector was made from alumina, which is sufficientlyresistant to acidified and hydrofluoric solutions. Otherwise suchsolutions may erode the injector tube and impact the finalresults.A peristaltic pump was used to introduce sample solutionsinto the ICP-AES at a flow rate of 2 mLmin–1. A cyclonic spraychamber with a cross-flownebulizerwas employed to allowhigherrates of sample introduction into the plasma. The detection wavelengths of Ni, Cr, Fe,Pb, Cd, Zn, Cu, Al, As, Mn, and Bi were 341.476, 357.869, 259.939, 217.000, 226.502, 202.548, 324.752, 308.215, 193.696, 257.610 and 306.766 nmrespectively.

Reagents and calibration

The single-element standards used for preparation of multi-element standards were Merck traceable to NIST standards. An intermediate standard solution containing 10 mg L-1of all the above mentioned analytes was prepared by mixing suitable aliquots of single- element stock solutions (Merck) containing Ni, Cr, Fe, Pb, Cd, Zn, Cu, Al, As, Mn, and Bi at 1000 mg L-1each, and appropriate dilution. This solution was further diluted in 0.5 mol L-1$HNO_3$to obtain a series of low concentration working standards (0-50mg L-1). Five-point calibration curves were prepared for elements and their slope was used to estimate the sensitivity in presence of yttrium as IS.

Statistical analysis

At the first part of the experiment we compared the survival fraction resulting from SRB-assay and data were statistically analysed using paired-t test, one-way analysis of variance (ANOVA) and Bonferonni post-hoc tests. To test the hypothesis that there were differences in heavy metal content we performed one-way analysis of variance (ANOVA) and Tukey multiple comparison tests. Significance level for hypotheses' rejection was set to p=0.05.

Results

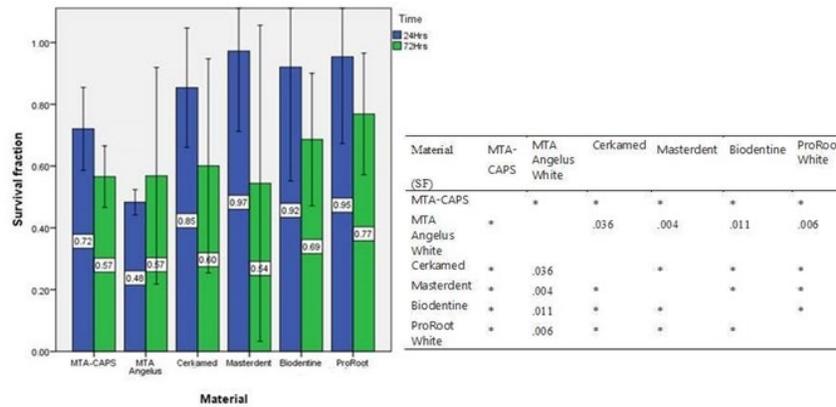The results of SRB assay are presented in Figure 1.

Figure 1: Survival fraction after incubation of cells with the cements for 24 and 72 hours and post-hoc p-values for 24 hours. No statistical significant difference was observed for 72 hours.

One-way analysis of variance determined that after 24 hours exposure, there was a significant difference between the groups ($p=0.02$), though there was no difference at 72 hours ($p=0.503$). Post-hoc analysis revealed that the cell survival fraction after exposure to MTA Angelus White was significantly reduced in comparison with all other groups ($p<0.05$). Within subjects' analysis was also performed between the 24 and 72 hours and showed that the 72 hours effect was significantly higher ($p<0.05$), except for Masterdent MTA ($p>0.05$). The concentrations of Ni, Cr, Fe, Pb, Cd, Zn, Cu, Al, As, Mn, and Bi in the tested materials are shown in Table 2.

Parameter	Value
RF generator	40 MHz, free-running
RF incident power	Optimized (1500 W)
Torch type	Fasset type
Injector, id	Alumina, 2.0 mm
Viewing mode	Axial
Auxiliary arfon flow rate	$0.50 \, l \, min^{-1}$
Nebulizer argon flow rate	$0.80 \, l \, min^{-1}$ (optimized)
Plasma gas flow rate	$15 \, l \, min^{-1}$
Spray chamber type	Cyclonic
Sample propulsion	Peristaltic pump, three channel
Sample uptake flow rate	$2 \, ml \, min^{-1}$
Detector	Segmented-array charge-coupled (SCD)

Detection of metals

	MTA-CAPS	MTA Angelus White	Cerkamed	Masterdent	Biodentine	ProRoot MTA White
Ni	ND	ND	ND	ND	ND	ND
Cr	ND	ND	ND	ND	ND	ND
Fe	200±0	ND	ND	200±0	ND	250±57
Pb	7600±4320	ND	ND	ND	ND	ND
Cd	ND	ND	ND	ND	ND	ND
Zn	1725±96	ND	ND	ND	ND	ND
Cu	ND	ND	ND	ND	ND	ND
Al	5650±870	7600±883	4900±650	775±95	ND	575±125
As	1675±125	ND	ND	ND	ND	ND
Mn	ND	ND	ND	ND	ND	ND
Bi	91800±8700	200600±12090	139200±7620	198980±17000	ND	201300±14500

Table 2: Parameters of ICP-AES and metal content analysis in the tested meterials.

For those elements where was available we hypothesized that the concentration levels would be equal for all the materials. The null hypothesis was retained for Fe ($p=0.111$), but was rejected for Al ($p=0.002$) and Bi ($p=0.005$). Since the null hypotheses were rejected, post-hoc analyses were performed. For the concentration of Al, MTA Angelus White differs from WhiteProRoot MTA ($p=0.002$) and Masterdent MTA ($p=0.04$). As for the Bi concentration MTA-CAPS differed from ProRoot White ($p=0.041$) and MTA Angelus White ($p=0.028$).

Discussion

The present study showed that the cytotoxicity of the tested materials depends on the time of exposure and the material. All materials were significantly more cytotoxic at 72h exposure than at 24h, except for Masterdent. MTA Angelus White had a significantly higher cytotoxic effect compared to the other cements tested at 24hours exposure whereas at 72hours no statistical significant difference was observed between materials. No previous information regarding the cytotoxicity of MTA CAPS, Cerkamed MTA+, and Masterdent MTA is available in the literature.

Moreover, the present study demonstrated that the cytotoxities of CerkamedMTA+, Masterdent, Biodentine and White ProRoot MTA were comparable. Previous studies have indicated that grey ProRoot MTA and MTA Angelus behave similarly in terms of cytotoxicity [29, 30]. Biodentine has also shown equivalent cytotoxic effect to MTA in previous studies[31, 32].

Cell culture models evaluate cytotoxicity by cell growth measurements, changes in membrane permeability, metabolic alteration and cell viability[13]. In the present study, we used MRC5 fibroblasts derived from an established cell-line, because of their well-defined cultural characteristics; this eliminated any variability due to different donors and gave the possibility to achieve reproducibility. The SRB assay is a fluorescent colorimetric assay, measuring the cellular protein content of cells, as it is bonded to the basic amino acids of cell proteins.

As cements are in direct contact with pulpal and periradicular tissues, their trace metal content may leach out into the body. Standard ISO 9917-1 recommends that the levels of leachable arsenic and lead in dental water-based cements should be <2 and 100 ppm, respectively. Arsenic (As) is an element, which has been used historically and fictionally. The toxicity of As is compound dependent[33].

Previous studies have examined either the As released from MTAs or their total arsenic content. The results are controversial, with Bramante et al[19]. and Schembri et al[26]. contending that the levels of As in some commercial forms of MTA are considerably in excess of those recommended by the ISO 9917-1 standard, but Duarte et al. (21) and De-Deus et al[23]. reporting negligible levels. In our experiment, As was detected only in MTA-Caps at the level of 1,675 ppm. Other studies using similar methodologies have also detected As in White ProRoot MTA [24, 34] and in White MTA-Angelus[35].

Lead (Pb) is an omnipresent toxic heavy metal, the mutagenic and genotoxic effects of which are still the subject of controversy[36]. This study found Pb only in MTA-Caps (7600ppm). The other tested materials were found to be free of Pb, which is in agreement with other studies[24, 37]. However, Camillieri et al[35]. detected Pb in Biodentine and White MTA-Angelus and Schembri et al[26].detected Pb in White ProRoot MTA.

Aluminum (Al) was detected in all the tested materials in this study, except Biodentine, at different concentrations. White MTA Angelus differs significantly from White ProRoot and Masterdent MTA regarding the concentration of aluminum. The U.S. EPA secondary maximum contaminant level (MCL) for total Al is 0.05-0.2 mg/L[38]. Considering that the amount of MTA used clinically is about 0.5 g, both the total amount of Al and the leaching of the metal into tissue fluids, is unlikely to exceed these values. Thus, the Al content could be considered safe from a clinical point-of-view.

The iron (Fe) content in MTA-Caps, Masterdent MTA and White ProRoot MTA was 200 ppm, 200 ppm and 250 ppm respectively, whereas no iron was detected in MTA Angelus White, Cerkamed MTA+ and Biodentine. Chang et al[24]. and Kum et al[34]. also detected Fe in White ProRoot MTA, but at significantly higher levels. The finding that some calcium silicate cements contain Fe could raise a concern that their application could cause tooth discoloration[39]. However, the correlation between the iron content in MTAs and tooth discoloration needs further investigation.

Zinc (Zn) was found only in MTA at the amount of 1725 ppm. Other researchers have also detected Zn in White ProRoot MTA, but at a lower level[24, 34].

Bismuth (Bi)is less toxic compared to other metals and showed no carcinogenic effect in long-term in vivo tests [40]. Bismuth oxide was added to Portland cement to enhance its radiopacity. ProRoot MTA has a 20% loading of bismuth oxide as reported by the manufacturer. The results of the present study confirm this claim, as the percentage of Bidetected in White ProRoot MTA was 20%. Biodentine contains zirconium oxide instead of bismuth oxide as radiopacifier.

In the present study, the tested cements were also scanned for nickel, chromium, cadmium, copper and manganese and none of these were detected. Interestingly, in previous studies many of these metals were detected in various silicate cements. The disagreement could be attributed to the different sample digestion methods, the method applied for the measurements and the detection limit of the equipment used. The digestion in the present study was performed using a mixture of strong acids (HNO_3, HCl, HF), because it has been proved that this mixture is suitable for the recovery of inorganic elements from the samples[41]. The detection limit of the apparatus can also be affected by a number of factors such as additional noise resulting from line overlap and ICP operating parameters[42].

The results of this study cannot be directly compared with the maximum allowable levels for As and Pb in water-based cements proposed by ISO 9917-1 due to the difference in the methods for extraction of trace elements used. ISO 9917-1 measures metal ions leached from dental cements using 2.4 mol L-1 of hydrochloric acid. The method applied in our study is slightly modified from the one used in ISO 11466 for extraction of the total metal amount from specimens. However, under in vivo conditions, trace metals in MTAs may be slowly released into body fluids, indicating that the selected method is probably less clinically relevant. Since the aim of this study was to compare very similar products, we chose this method due to its sensitivity and its ability to differentiate the minute differences of trace metals in each material tested.

Conclusions

This study indicated that the silicate-based cements tested demonstrated similar cytotoxic profiles, except for White MTA Angelus which showed a significantly higher effect. In parallel, all cements except for Biodentine, contain traces of metals, although in concentrations below the safety limits.

Acknowledgements

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