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Cytotoxicity and genotoxicity of pulp capping materials in two cell lines

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Abstract

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Aim The aim of this study was to evaluate the cytotoxicity and genotoxicity of the new castor oil bean cement (COB) material in comparison to commonly used pulp capping materials.

Methodology Specimens of COB, calcium hydroxide (Hydro C), and mineral trioxide aggregate (white and gray MTA) were extracted in culture medium (91.6 mm^2 sample surface mL^{-1}). Transfected human pulp cells (tHPCs) were exposed to dilutions of the extracts for 1 h, and the generation of reactive oxygen species (ROS) was determined by flow cytometry (FACS) using H_2DCF-DA as a dye. Survival of tHPCs was measured photometrically using a crystal violet assay after a 24-h exposure period. Genotoxicity as indicated by the formation of micronuclei in V79 cells, and the modification of the normal cell cycle by extracts of the materials was analysed by FACS.

Results Clear cytotoxic effects were detected only with extracts of Hydro C under the current experimental conditions. The two MTA preparations induced an insignificant reduction in the number of cells. In contrast, the extracts of COB slightly induced cell proliferation. Extracts of Hydro C caused a twofold increase in ROS production, whilst the other tested materials were ineffective. An increase in the number of micronuclei was not detected with any material tested; Hydro C slightly increased the number of cells in G1 and G2.

Conclusions The COB and the two MTA preparations did not negatively influence cell survival or ROS production and may thus be further considered for pulp capping studies.

Keywords: cytotoxicity, genotoxicity, pulp capping materials, reactive oxygen species.

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Introduction

One of the objectives of operative dentistry is to maintain pulp health in compromised teeth, thus reducing the need for root canal treatment and the potential for unwanted sequelae such as tooth loss. Commonly used methods for this purpose are direct pulp capping and pulpotomy, which consist of placement of biocompatible materials and bio-inductors on the exposed pulp tissue to preserve its health and stimulate repair by mineralized tissue formation (Dominguez *et al.* 2003, Accorinte *et al.* 2008). New materials are now available that claim to be biocompatible, have antibacterial properties, act as healing inductors and provide appropriate sealing. Thus, these materials may improve the higher longevity of the

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tooth after direct pulp capping and pulpotomy (Dominguez *et al.* 2003, Accorinte *et al.* 2008). The success of such treatments depends, besides other factors, on the applied material (Cohen & Hargreaves 2006). Several materials are commonly recommended for this purpose such as calcium hydroxide and more recently mineral trioxide aggregate (MTA) (Dominguez *et al.* 2003, Camilleri & Pitt-Ford 2006).

Calcium hydroxide has been the material of choice for pulp capping and pulpotomy. It is assumed that the material leads to initial change that causes undifferentiated cells within the pulp to differentiate into odontoblasts, which then form a hard tissue barrier at the pulp exposure site. The formation of reparative dentine in response to calcium hydroxide may not be due to the bioinductive capacity of the material but due to the result of a defense mechanism by the pulp induced by the irritant nature of calcium hydroxide (Goldberg *et al. 2003,* Almushayt *et al.* 2006).

The MTA has antibacterial properties in an aqueous environment due to its high pH (12.5) and good biocompatibility in cell cultures (Hauman & Love 2003). Thus, it has been used as root-end filling material in apical surgery, as sealing material and for pulp capping and pulpotomy (Dominguez *et al.* 2003, Camilleri & Pitt-Ford 2006). In comparison to calcium hydroxide, MTA induces the formation of a hard tissue barrier at the pulp exposure site, but evokes a less prominent inflammatory reaction (Hauman & Love 2003). Clinically, similar positive results have been observed for MTA and for calcium hydroxide preparations (Accorinte *et al.* 2008).

Recently, a material based on a polymer derived from the castor oil plant (*Ricinus communis*) was introduced into endodontics. This material, castor bean polyurethane cement (COB), is composed of 81–96% triglyceride of ricinoleic acid, and is considered a natural polyol containing three hydroxyl radicals. This cement has been reported to have antibacterial properties, is compatible with living connective tissues, has the potential to facilitate tissue healing, shows good mechanical properties, and is available at low cost (Carvalho *et al.* 1997, Barros *et al.* 2003). It has been successfully used as a root-end filling material in apical surgery, and as a sealer for root fillings (Pascon *et al.* 2001). Due to these positive characteristics, the material is considered to be an excellent candidate for use in pulp capping.

Ideally, pulp capping materials should be biocompatible and have satisfactory physico-chemical properties. As these materials will be in direct contact with pulp tissue for long periods of time, the biocompatibility is of particular importance. A biocompatible material should not only promote tissue repair, but should also aid or stimulate the reorganization of injured structures (Carvalho *et al.* 1997, Mantellini *et al.* 2003).

For the determination of biocompatibility of dental materials a large number of methods have been recommended, with the analysis of cellular reactions *in vitro* generally considered to be the initial approach (Schmalz 1994). This allows for the basic biological characterization of a material and for analysis of the underlying cellular mechanisms. Cellular actions of interest are cellular toxicity (cytotoxicity) and the influence of sublethal concentrations on heritable DNA damage (genotoxicity). Recently, the generation of elevated levels of reactive oxygen species (ROS) by dental resin monomers has been related to the induction of cytotoxicity, genotoxicity, and to changes in the normal cell cycle (Schweikl *et al.* 2006).

Therefore, the purpose of this study was to evaluate the cytotoxicity and genotoxicity of the new COB material in comparison to commonly used pulp capping materials. The cytotoxicity of extracts of the various materials was measured by staining surviving cells with crystal violet, and analyzing the generation of ROS as recorded by flow cytometry (FACS) using transfected human pulp cells (tHPCs). The genotoxicity was determined by the formation of micronuclei in V79 cells, and related effects of the materials on the normal cell cycle were detected by FACS.

Material and methods

Preparation of extracts

The following materials were used: Calcium hydroxide cement (Hydro C; Dentsply, São Paulo, SP, Brazil), gray mineral trioxide aggregate (gray MTA; Angelus, Londrina, PR, Brazil), white mineral trioxide aggregate (white MTA), and castor oil bean cement (COB) (*Ricinus communis*; Poliquil, Araraquara, SP, Brazil). Vitrebond (3M ESPE Dental Products, St. Paul, MN, USA) was used as a reference material. A list of the components of the test materials is presented in Table 1. Samples of the materials were prepared in Teflon rings (5 mm in diameter, 2 mm high). Vitrebond specimens were light-cured for 40 s (780 mW cm^{-2}) from each side. Seven material specimens were transferred immediately after mixing into one insert (Millipore (Billerica, MA, USA), 0.4 μm filter, 30 mm diameter) of a 6-well plate. The test specimens were covered with 3 m cell culture

Table 1 Principal components of the pulp capping materials tested

Material	Components
Calcium hydroxide cement (Hydro C) (Dentsply, São Paulo, SP, Brazil) Lot no: 328439	Calcium tungstate; zinc oxide; disalicylate ester of 1,3 butylene glycol and calcium phosphate
Gray mineral trioxide aggregate (MTA) (Angelus, Londrina, PR, Brazil) Lot no: 5494	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, bismuth oxide, iron oxide, calcium oxide
White mineral trioxide aggregate (MTA) (Angelus, Londrina, PR, Brazil) Lot no: 822, 7122	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, bismuth oxide, iron oxide, calcium oxide
Castor oil bean cement (COB) (Poliquil, Araraquara, SP, Brazil) Lot no: 14001011	Liquid Polymer: Vegetable polymer extracted from oily *Ricinus communis* Powder: Calcium carbonate
Vitrebond (3M ESPE Dental Products, St. Paul, MN, USA) Lot no: 20070205	Powder: radiopaque, ion-leachable fluoroaluminosilicate glass powder Liquid: modified polyacrylic with pendant methacrylate groups, HEMA (2-hydroxyethylmetacrylate) water and photoinitiator

medium [MEMα supplemented with 10% fetal bovine serum (FBS), geneticin and penicillin/streptomycin] and incubated in the dark for 24 h at 37 °C. Thus, extracts of the test specimens were prepared at a ratio of 91.6 mm^2 sample surface area m^{-1} cell culture medium. After a 24-h extraction period, these original extracts (1 : 1) were then serially diluted in cell culture medium prior to testing.

Cytotoxicity testing

Primary human pulp-derived cells were originally transfected with a plasmid containing coding sequences of the SV40 large T-antigen. This resulted in the establishment of a cell line with an extended of life span (Galler *et al.* 2006). These tHPCs were routinely cultivated in MEMα supplemented with 10% FBS, geneticin and penicillin/streptomycin at 37 °C, and 5% CO_2 as described (Galler *et al.* 2006). In each well of a 96-well plate, 8×10^3 cells were seeded and incubated for 24 h at 37 °C. Then, the cell cultures were exposed to 200 μL of original extracts of the test materials and serial dilutions of these extracts. Discarding the exposure medium stopped the exposure of the cell cultures after 24 h. Then, cell survival was determined using a crystal violet assay. Cell cultures were fixed with 1% glutaraldehyde, washed twice with phosphate-buffered saline (PBS), and stained with crystal violet (0.02% in water). The amount of crystal violet bound to the cells was dissolved with 70% ethanol, and optical densities were measured at 600 nm in a multi-well spectrophotometer (Infinite F200; TECAN, Crailsheim, Germany). Four replicate cell cultures were exposed to each of the serial dilutions of material extracts in three independent experiments. Optical density readings were normalized to untreated control cultures (=100%), and differences between median values were statistically analysed using the Mann–Whitney *U*-test (SPSS 15.0; SPSS, Chicago, IL, USA) for pairwise comparisons among groups at the 0.05 level of significance.

Measurement of reactive oxygen species

The generation of ROS was measured using the oxidation-sensitive fluorescent probe 2′7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA). Intracellular esterase activity results in the formation of DCFH, a nonfluorescent compound which emits fluorescence when it is oxidized to DCF-DA (Demirci *et al.* 2008). tHPCs (2×10^5) were seeded into each well of a six-well plate and incubated for 24 h at 37 °C. The cells were then exposed to serial dilutions of the various material extracts (2 mL per well) and pre-incubated for 30 min at 37 °C. Next, 2 μL DCFH-DA (10 mmol L^{-1}) were added to the cell cultures for 30 min at 37 °C. Untreated cell cultures were used as negative controls. One well was used for each concentration and exposure was stopped after 1 h. Then, cell cultures were detached with Accutase (PAA; Laboratories GmbH, Pasching, Austria), resuspended in 250 μL CMF–PBS, and DCF fluorescence was determined by FACS (BD

FACSCanto, BD Biosciences, Heidelberg, Germany) at an excitation wavelength of 495 nm and an emission wavelength of 530 nm (FL-1). Mean fluorescence intensities were obtained by histogram statistics using the BD FACSDiva software. At least four independent experiments were performed with each compound. Mean fluorescence intensities were normalized to untreated control cultures (=1.0), and differences between median values were statistically analysed using the Mann–Whitney *U*-test for pairwise comparisons among groups at the 0.05 level of significance.

Micronucleus test *in vitro*

V79 Chinese hamster fibroblasts (3×10^5 cells) were cultivated on microscopic glass slides in 4 mL MEMα supplemented with 5% FBS, and penicillin/streptomycin for 24 h at 37 °C and 5% CO_2. Test materials were extracted in V79 cell culture medium as described above. Then, the cell cultures were exposed to dilutions of extracts of Hydro C, gray MTA, COB and Vitrebond for 24 h. These dilutions were either mildly cytotoxic in V79 cells or the most concentrated extracts available under the present experimental conditions were used. Ethyl methanesulfonate (EMS) served as a positive control, and the number of micronuclei was determined microscopically in 1000 cells/slide of two parallel cultures (slides) per concentration as previously described (Schweikl *et al.* 2001). At least four slides derived from two independent experiments were analysed, and differences between median values were statistically analysed using the Mann–Whitney *U*-test for pairwise comparisons among groups at the 0.05 level of significance.

Cell cycle analysis

V79 cells were incubated at an initial cell density of 0.25×10^6 per cell culture plate (150 mm in diameter) at 37 °C and 5% CO_2. Subconfluent monolayer cell cultures were then exposed to two dilutions of extracts of Hydro C (1 : 8 and 1 : 16), MTA (1 : 1 and 1 : 2), and COB (1 : 1 and 1 : 2). TEGDMA (1.5 mmol L^{-1}) was used as a positive control. The distribution of the number of cells among the three phases (G1, S, G2) of the cell cycle was determined by FACS as previously described (Schweikl *et al.* 2007). Statistical analyses of differences between the DNA content of cells in G1, S or G2 phase in untreated and treated cell cultures were performed using the Mann–Whitney *U*-test for pairwise comparisons among groups at the 0.05 level of significance.

Results

Cytotocixity of pulp capping materials and ROS production

Original extracts of Hydro C reduced survival rates of tHPC to 20.5%, and 9.8% of exposed cells survived after exposure to a 1 : 2 dilution of the original extract (Fig. 1). Extracts of Hydro C were significantly more toxic to pulp cells than extracts of COB and mineral trioxide aggregate (gray and white MTA) samples for both original extracts and their dilutions up to 1 : 8 ($P < 0.001$). Yet, it appeared as if the undiluted extracts and a 1 : 2 dilution of gray MTA samples caused a slightly more pronounced reaction in tHPC than white MTA. While this difference between gray and white MTA was not statistically significant, the undiluted extract of COB actually significantly increased cell numbers to 105.2% ($P \leq 0.05$). Vitrebond was found to be the most toxic material tested in this experiment, since the difference in survival rates between Vitrebond and all other materials was statistically significant for all dilutions ($P \leq 0.01$). Since the pH values of all extracts varied between 7.0 and 8.5, cytotoxicity was apparently not related to differences in pH values of the extracts. Summarizing the results for the crystal violet tests, the toxicity of the test materials can be ranked in the following order from the most toxic to the least toxic preparation: Vitrebond > Hydro C >> white MTA = gray MTA >>> COB.

The generation of DCF fluorescence, which is indicative of ROS production in tHPC, was determined after a short exposure period of 1 h to extracts of the pulp capping materials (Fig. 2). The original extracts as well as dilutions (1 : 2) of Hydro C led to a twofold increase in ROS production. In contrast, increased ROS production was not detected in any extracts of gray and white MTA or COB ($P > 0.05$). On the other hand, original extracts of Vitrebond increased ROS production about sevenfold (Fig. 2).

Formation of micronuclei and influence of pulp capping materials on the cell cycle

The formation of micronuclei by extracts of the pulp capping materials was analysed in V79 cell cultures (Fig. 3). EMS, which was used as a positive control, increased the number of micronuclei in treated cultures about sevenfold compared to those detected in untreated controls. In contrast, no increase in the number of micronuclei was detected with the dilutions of Hydro

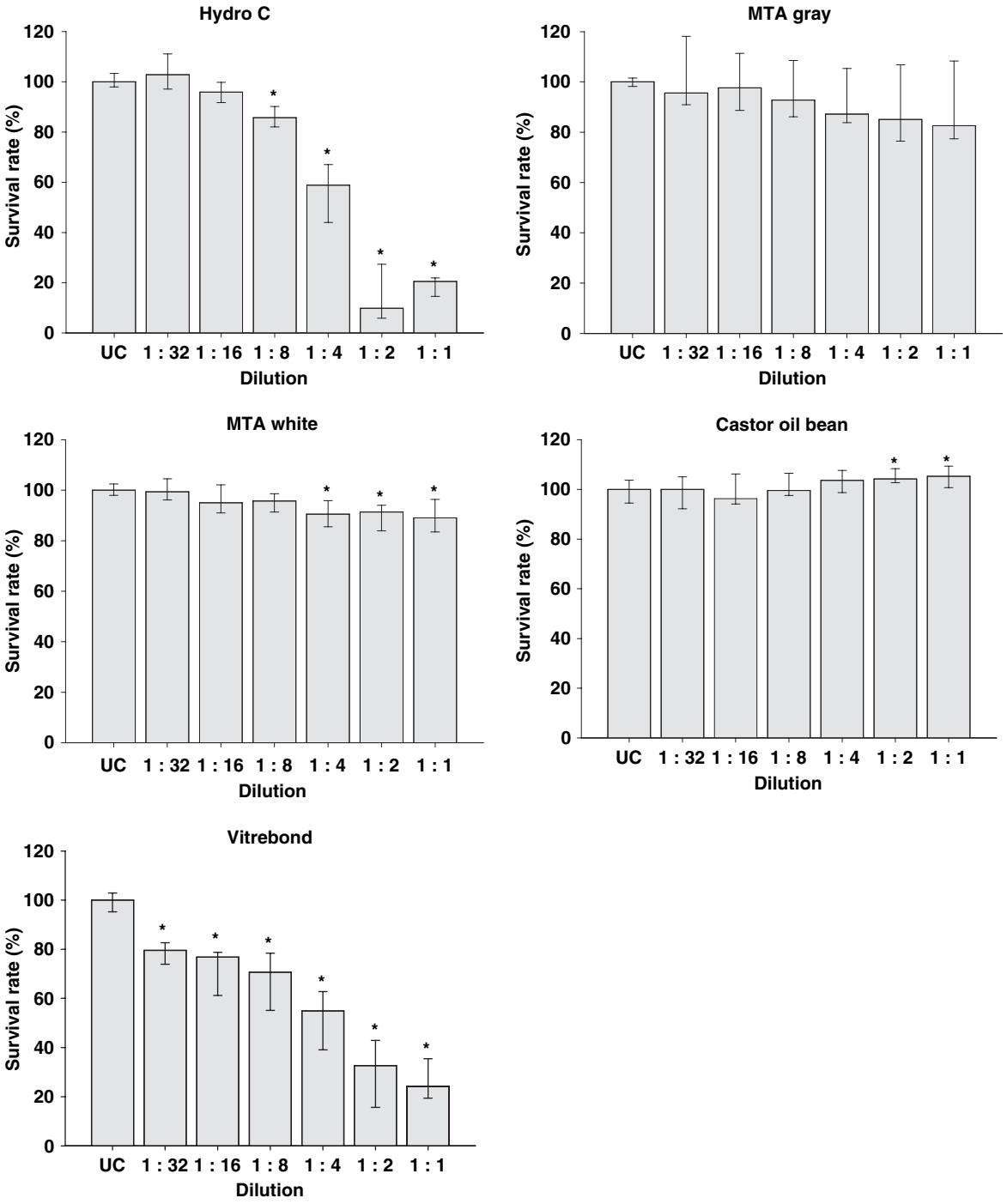

Figure 1 Cytotoxicity of pulp capping materials in tHPC after exposure to extracts. Original extracts (1 : 1) were serially diluted with cell culture medium as indicated. The cell cultures were exposed for 24 h, and cellular survival in treated and untreated cell cultures was determined in quadruplicate in three independent experiments (n = 12). Bars represent medians plus 25% and 75% percentiles. Statistically significant differences between untreated and treated cell cultures are indicated by asterisks. UC, untreated controls.

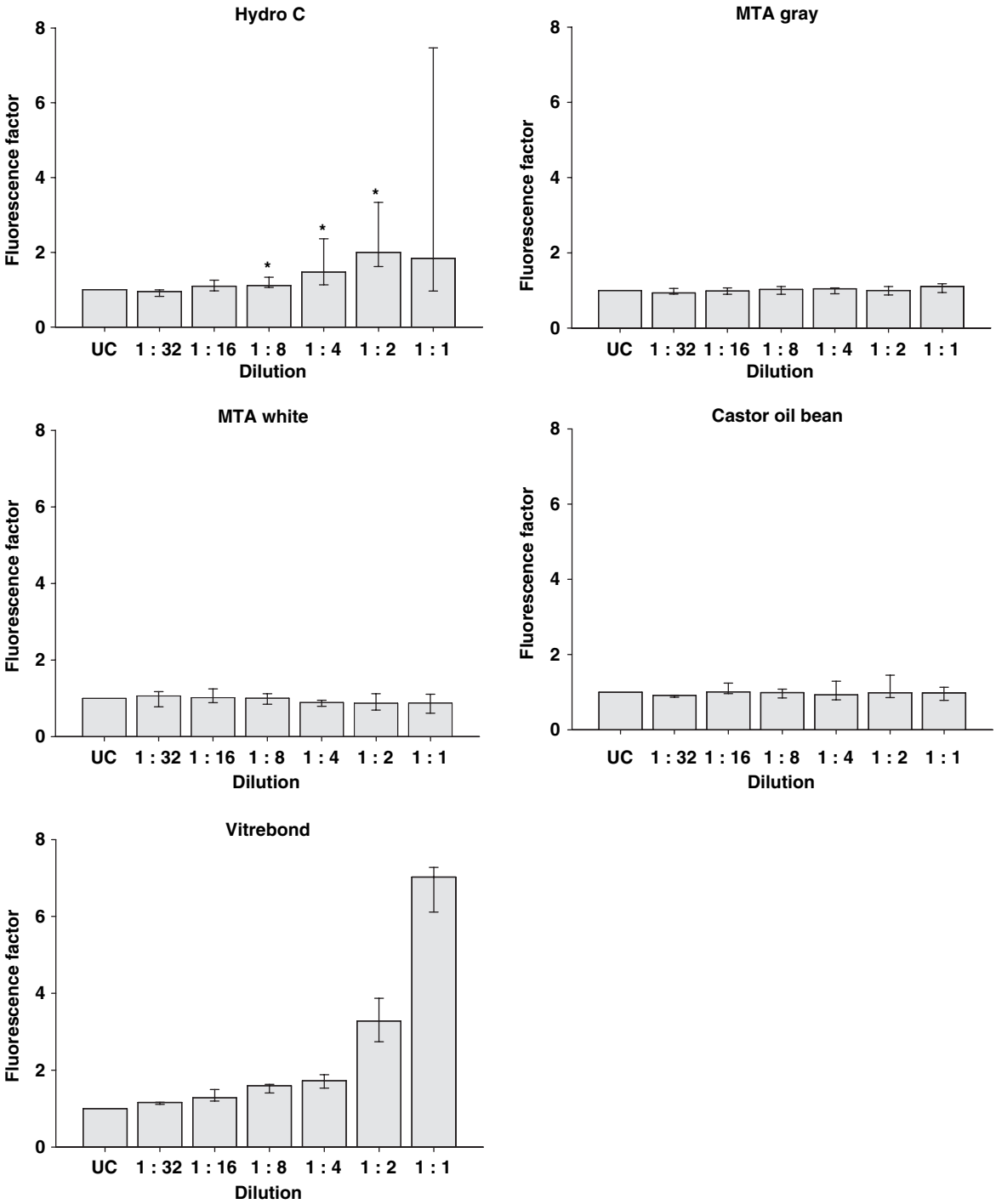

Figure 2 Generation of ROS in tHPC after exposure to pulp capping materials. The production of ROS was measured using the oxidation-sensitive fluorescent probe 2′7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA). The cell cultures were exposed to increasing dilutions of pulp capping materials in cell culture medium for 1 h. Mean fluorescence intensities were normalized to untreated control cultures (UC = 1.0). Bars represent medians (25% and 75% percentiles) calculated from four independent experiments (n = 4). Statistically significant differences between untreated and treated cell cultures are indicated by asterisks.

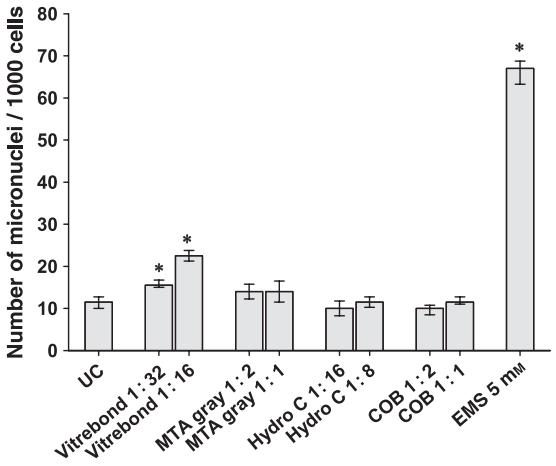

Figure 3 Induction of micronuclei in V79 cells after exposure to pulp capping materials. The number of micronuclei caused by the pulp capping materials were obtained from four to six treated cell cultures (n = 4–6), and bars represent medians (25% and 75% percentiles). Median numbers of micronuclei were also calculated for untreated controls (UC), and 5 mmol EMS (n = 20). Statistically significant differences between untreated and treated cell cultures are indicated by asterisks.

C, gray MTA and COB extracts tested here ($P > 0.05$) (Fig. 3). Diluted extracts of Vitrebond specimens increased the number of micronuclei about 2.5-fold ($P < 0.05$). Higher concentrations of Hydro C and Vitrebond tested cytotoxic in V79 cells, and micronuclei could not be counted.

Extracts of the various materials were also analysed for their influence on the cell cycle of V79 cells. About 33% of the cells in untreated controls were found in the G1 phase of the cell cycle, and 12% of the cells were detected in G2 phase (Fig. 4). The relatively high percentage (55%) of cells in S phase indicated a high proliferative activity of V79 cells. TEGDMA (positive control) caused a delay of the V79 cell cycle. More than 45% of the V79 cells in cell cultures treated with 1.5 mmol L^{-1} TEGDMA arrested in G2, and only 10% of the cells were found in G1 (Fig. 4). Dilutions of Hydro C slightly, but significantly, increased the number of cells in G1 and G2 (1 : 8) compared with untreated control cultures. These minimal changes were associated with a small decrease in the number of cells in S phase (Fig. 4). Extracts of gray MTA (1 : 1) caused a minor increase in the number of cells in G2 phase related to a slight decrease in the cell population in S phase. Extracts of COB (1 : 1 and 1 : 2), however, did not evoke any statistically significant differences in the number of cells in G1, S and G2 in treated cultures compared to those seen in untreated control groups ($P > 0.05$).

Discussion

Cytotocixity of pulp capping materials and ROS production

In the present study, extracts of Hydro C reduced the number of tHPC to approximately 10%, and thus was

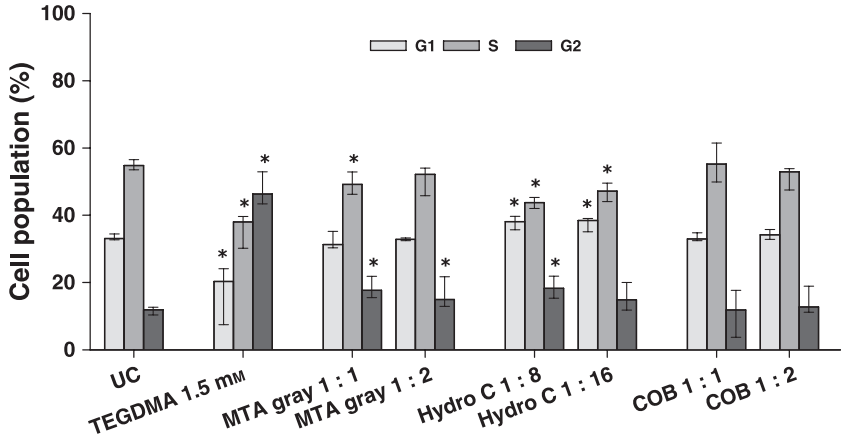

Figure 4 Cell cycle analysis of V79 cells. The distribution of V79 cells between the three phases of the cell cycle (G1, S and G2) is presented after exposure to extracts of pulp capping materials for 24 h. The percentages of cell numbers in each phase were calculated from individual histograms in four independent experiments (n = 4). Bars represent medians (25% and 75% percentiles), and asterisks indicate statistically significant differences of cell populations in G1, S or G2 from corresponding untreated cell cultures.

the material with the highest toxicity within the series tested. In contrast to these results, it has been reported that cell culture medium conditioned with $Ca(OH)_2$ cement did not decrease the number of human pulp cells (Cavalcanti *et al.* 2005). The discrepancy between these data and the present results may be explained by differences in experimental procedures such as sample preparation, extraction and cell culture methods. On the other hand, Hydro C was much more cytotoxic than the MTA preparations when tested under the current conditions. This corresponds to reports on the cytotoxicity of Portland cement, which is virtually the same material as MTA. No cytotoxicity was detected with Portland cement up to a 72-h exposure period, but a calcium hydroxide preparation drastically reduced cell viability (Min *et al.* 2007). It was also observed that white MTA evoked higher cell proliferation than a $Ca(OH)_2$ cement in human pulp cells (Takita *et al.* 2006). The present result suggests that Hydro C may interfere with pulp healing under certain circumstances based on the pronounced cytotoxicity observed under the present experimental conditions.

The MTA was repeatedly ranked as nontoxic or the least cytotoxic root canal cement in various cell lines and test systems (Osorio *et al.* 1998, De-Deus *et al.* 2005, Souza *et al.* 2006, Rezende *et al.* 2007). However, some authors reported that MTA may be able to reduce cell viability after short exposure periods (De-Deus *et al.* 2005, Guven *et al.* 2007). On the other hand, there is also evidence that MTA induced the expression of bone morphogenetic protein (BMP)-2 and an osteogenic phenotype (Bonson *et al.* 2004, Guven *et al.* 2007). Because of the reduced expression of cytokines in pulp tissues exposed to MTA, an anti-inflammatory effect of the material was suggested (Silva *et al.* 2008).

The MTA is commercially available in two colours, but most studies evaluated the gray preparation since white MTA has only been marketed relatively recently. These materials contain differences with respect to their concentrations of aluminium, magnesium and iron compounds. White MTA lacks the aluminoferrite phase that imparts the gray colour to gray MTA (Camilleri & Pitt-Ford 2006). It has been reported that oral epithelial cells and cementoblasts may proliferate better on the surface of white MTA than gray MTA (Oviir *et al.* 2006). In the present study, no significant differences were detected between the gray and white MTA preparations. Similarly, no such differences were observed using different cell culture methods and exposure periods of up to 28 days (Camilleri *et al.* 2004, Ribeiro *et al.* 2005). Likewise, the induction of apoptosis was not detected in odontoblast-like cells (MDPC-23) and undifferentiated pulp cells (OD-21) (Moghaddame-Jafari *et al.* 2005).

The COB is a natural polyurethane resin obtained by polymerization of the polyester polyol derived from *Ricinus communis.* COB or RCP (*Ricinus communis* polyurethane) was originally developed as a biomaterial for bone repair and regeneration after local bone damage. Animal studies have shown that RCP promoted fibroblastic neoformation, which was progressively replaced by bone around and inside the porosities of the biomaterial. Late inflammatory reactions and systemic toxic effects were not detected (Kojima *et al.* 1995). It was reported that RCP allowed for the neoformation of bone after implantation into rat alveolus. Nevertheless, retardation in the wound-healing process was observed in the presence of RCP in the cervical third of rat alveolus (Carvalho *et al.* 1997). Yet, it was originally suggested that the material is compatible, because of its progressive integration into the bone tissue (Carvalho *et al.* 1997). The generation of mineralized bone matrix in close contact with the RCP surface was repeatedly demonstrated (Barros *et al.* 2003). RCP was also coated with calcium carbonate or calcium phosphate, and surgically implanted in rabbit femurs. It was observed that osseointegration was increased by RCP-$CaCO_3$ after 16 weeks, and by RCP-$Ca_3(PO4)_2$ after 8, 12 and 16 weeks. It appeared as if the process of osseointegration was already completed on RCP-$Ca_3(PO4)_2$ after 8 weeks. Therefore, it was suggested that the addition of calcium phosphate improved the biocompatibility of the RCP. Compared with hydroxyapatite, RCP was considered a bioinert rather than a bioactive material (Barros *et al.* 2003). Corresponding results were obtained in *in vitro* studies on cell adhesion and proliferation on the same RCP materials (Beloti *et al.* 2003). To further promote tissue integration and stimulate tissue regeneration, RCP was coated with alkaline phosphatase (ALP) and incubated in synthetic body fluid (SBF) before responses of bone marrow stem cells cultured in osteogenic medium were analysed. Although the combination of RCP–ALP was cytotoxic for unknown reasons, it was found that cell viability was not affected by RCP, and RCP–ALP after incubation in SBF. However, bone-like nodule formation was not observed. It was suggested that the incorporation of ALP to the RCP followed by SBF incubation could be a useful alternative for improving the biological properties of the RCP (Beloti *et al.* 2008). In the present study, cytotoxicity of COB was not

detected in tHPC. In contrast, a slight increase in cell numbers compared to untreated control cultures was observed, suggesting that this material might be able to promote proliferation. Based on the *in vitro* and *in vivo* studies discussed above, the results add further evidence that COB should be considered a promising material for further pulp capping studies.

A large number of analyses showed the high cytotoxicity of Vitrebond using different evaluation methods (Schuster *et al.* 2001, Galler *et al.* 2005). On the other hand, it was reported that Vitrebond allowed for pulp healing and the formation of a calcified barrier at the exposure site (Souza-Costa *et al.* 2003). However, it should be noted that these experiments were normally performed on healthy pulp tissues, and the use of the material for direct pulp capping was not recommended by the manufacturer.

Data on the increased generation of ROS may provide insight into one possible mechanism of cellular toxicity. In the present study, neither gray and white MTA, nor COB increased the amounts of ROS after a 1-h exposure period. This is in line with the lack of cytotoxicity observed for these materials under the current experimental conditions. These findings also correspond to a recent report on the lack of ROS production in macrophages exposed to MTA (Rezende *et al.* 2007). Here, Hydro C caused only a small increase in ROS levels, but extensive cytotoxicity was observed. This effect is different from the reactions observed with, for instance, Vitrebond and the resin monomer TEGDMA (Demirci *et al.* 2008). Thus, cytotoxicity of materials like Vitrebond and resin monomers appears to be closely associated with ROS production whereas cytotoxicity of materials like Hydro C is based on a different mechanism.

Influence of pulp capping materials on the formation of micronuclei and the cell cycle

The *in vitro* micronucleus test is a suitable method for testing the genotoxic potential of chemical substances. It has also been previously used to check for the induction of clastogenic effects caused by resin monomers or dental materials (Schweikl *et al.* 2001, Demirci *et al.* 2008). In the present study, Hydro C and gray MTA did not increase the number of micronuclei in V79 cells. Likewise, no genotoxic effects were detected with COB. Our findings with MTA are in agreement with studies using the single cell gel (comet) assay to evaluate DNA damage. Furthermore, the clear genotoxic effect observed here with Vitrebond is in line with data reported in the literature as well (Ribeiro *et al.* 2006a,b).

It has been repeatedly shown that monomers of dental composite resins are able to influence the normal cell cycle, probably as a result of DNA damage (Schweikl *et al.* 2007). Therefore, this biological endpoint was included in the present study. Here, COB had no influence on the cell cycle of V79 cells. The significant increase in the number of cells in G1 and G2 phase caused by a 1 : 8 dilution of the Hydro C extract can be correlated with a decrease in cell survival as detected by the crystal violet assay. A slight influence of gray MTA on the cell cycle was observed here. Interestingly, it has been previously reported that MTA induced a significant decrease in the number of pulp cells in G1 phase and a slight but significant increase in S and G2 phase, suggesting that MTA may have a positive effect on the regeneration of the dentine-pulp complex *in vivo* (Moghaddame-Jafari *et al.* 2005).

Conclusions

Cellular reactions to the pulp capping materials analysed here strongly depended on the material tested. It appeared as if the cytotoxicity of the calcium hydroxide preparation Hydro C was not related to an increased generation of ROS. Therefore, it can be assumed that the cytotoxicity of this preparation is modulated by a mechanism different from, for instance, dental resin materials. COB as well as the MTA preparations were the materials showing the least cytotoxicity. Moreover, no increase in ROS production, formation of micronuclei, or any influence on the mammalian cell cycle could be detected. This lack of adverse cellular effects of COB in the present study combined with the promising reports from *in vivo* studies on osseointegration make this material a promising candidate for future pulp capping studies.

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