

***In vitro* evaluation of the cytotoxicity of ProRoot MTA and MTA Angelus**

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Abstract: The purpose of the present *in vitro* study was to compare the cytotoxic effect of two commercially available brands of mineral trioxide cement (ProRoot MTA and MTA Angelus), modified zinc oxide-eugenol cement (SuperEBA) and resin-modified glass ionomer cement (Vitrebond) using rat pulp cells (RPC-C2A) and human lung fibroblasts (MRC-5). The cells were cultured in typical culture conditions and exposed to the tested materials by adaptation of insert wells. The cytotoxic effect was recorded at two observation periods (24 and 72 h) by using a colorimetric assay of tetrazolium reduction (XTT method) in reference to controls. Overall, the degree of cytotoxic effect in ascending order was ProRoot MTA – MTA Angelus < SuperEBA < Vitrebond. Both MTA materials tested exerted mild suppression of cellular mitochondrial activity and may be characterized as biologically inert materials. (J. Oral Sci. 50, 397-402, 2008)

Keywords: mineral trioxide aggregate; cytotoxicity; XTT method; pulp cells.

Introduction

Mineral trioxide aggregate (MTA) has been proposed for use as root-end filling material (1,2), root or furcal perforation repair material (3) and apexification and obturation of the root canal system (4,5). Additionally, MTA is an effective pulp capping material able to stimulate reparative dentine formation by the stereotypic defensive mechanism of early pulpal wound healing (6,7). The material is based on Portland cement except for the addition of bismuth oxide to improve radiopacity. In the dental market, two commercial brands are available - the ProRoot MTA and the MTA Angelus, which was recently introduced. Although several studies reported the biological effects of ProRoot MTA, only a small amount of scientific information has been published on the comparative evaluation of the biocompatibility of ProRoot MTA and MTA Angelus (8-10). Specifically, no data exist on MTA Angelus and its biocompatibility on cells of dental origin, such as pulp cells.

During routine clinical procedures, it is possible to expose the pulp tissue and proceed to pulp capping to preserve pulp vitality and promote healing and function. Several factors influence the pulp capping procedure, such as age, stage of root formation, size of exposure, microbial contamination etc.

Over the years, a number of materials have been proposed for pulp capping including zinc oxide-eugenol cements, calcium hydroxide cements and conventional or resin modified glass ionomer cements (RMGICs), which are

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characterized by improved physical and mechanical properties compared to conventional glass ionomer cements (11). Recently developed materials, such as MTA, have also been proposed as effective pulp capping materials based on their ability to stimulate pulp tissue repair and promote dentin bridge formation (6,12,13).

Studies with cell cultures may offer a significant tool to improve our knowledge of possible toxic effects of materials and for predicting these effects on humans. Furthermore, *in vitro* tests are simple to perform, repeatable, cost-effective, relevant and suitable as an alternative to *in vivo* experiments. The *in vitro* assays usually estimate cell numbers after exposure to the tested materials and reference to control cells produces a survival fraction, i.e. the percentage of viable cells at the end of the *in vitro* experiment.

The purpose of the present study was to evaluate the cytotoxic effect of two commercial brands of MTA (ProRoot MTA and MTA Angelus), a zinc oxide-eugenol cement (SuperEBA) and a resin modified glass ionomer cement (Vitrebond) using a cell viability assay for mitochondrial dehydrogenase activity in rat pulp cells and human lung fibroblasts.

Materials and Methods

Cell lines and culture conditions

Human fibroblasts (MRC-5; obtained from Theagenion Cancer Tissue Bank) and rat pulp cells (RPC-C2A; gift from Professor S. Kasugai, Department of Pharmacology, Faculty of Dentistry, Tokyo Medical and Dental University, Japan) were grown as monolayer cultures in T-75 flasks (Costar/Corning), subcultured twice a week at 37°C in an atmosphere containing 5% CO₂ in air and 100% relative humidity. The culture medium was Dulbecco's modified Eagle medium (DMEM, Gibco, Glasgow, UK), supplemented with 10% fetal bovine serum (FBS, Gibco, Glasgow, UK), 100µg/ml streptomycin and 100 IU/ml penicillin.

Adherent cells at a logarithmic growth phase 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 min at 37°C. As determined by hemocytometry, cells were plated on 12-well plates (Costar-Corning, Cambridge) at a density of 30,000 cells per well in complete medium and were placed in the incubator for 24 h to obtain exponential cell growth.

Preparation of test materials

The tested materials were: white ProRoot MTA (Dentsply, York, PA, USA); white MTA Angelus (Angelus, Londrina, Brazil); SuperEBA (Bosworth Co., Skokie, IL,

USA); and Vitrebond (3M/ESPE, St. Paul, MN, USA). All materials were prepared according to the manufacturer's instructions and placed at the bottom of transwell insert wells, with a membrane pore diameter of 0.4µm that fitted in the 12-well microplates. After setting, the insert wells were UV-irradiated (180 J/cm²), placed into the culture wells and incubated for 24 or 72 h. Six wells per material were prepared. In controls, cells were cultured in 12-well plates with transwell inserts but without any material specimen. After completion of the exposure time, the insert wells were removed and cell numbers were estimated by the 2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) assay.

XTT assay

The XTT assay was performed as previously described (14,15). Briefly, 500µl of a mixture (100:1) of XTT (1 mg/ml) (Sigma Chemical Co, St. Louis, MO, USA) with 10 mM menadione (MEN) were added to the wells already containing 2 ml of cells in culture medium and plates were incubated for 4 h at 37°C. Absorbance was read in an Elisa plate reader (Anthos 2001) at 450 nm subtracting the background measurement of 620 nm.

The test optical density (OD) value was defined as the mean absorbance of each individual well, minus the blank value ('blank' is the mean optical density of background control wells). Results were expressed as survival fraction following the equation (OD_{test}/OD_{control}) × 100. The mean optical density of the control wells (where empty insert wells were applied) was set to represent 100% viability.

The statistical analysis of the results was performed by Kruskal Wallis test, followed by Mann Whitney test with Bonferroni correction ($P < 0.05$).

Results

Each experiment was repeated at least twice and the results of typical experiments are presented in Tables 1 and 2. Representative photos of cells after exposure to the tested materials are shown in Fig. 1. Overall the ranking order of the materials tested was Vitrebond > SuperEBA > ProRoot MTA – MTA Angelus.

Vitrebond showed the highest cytotoxic effect and the mitochondrial dehydrogenase activity decreased significantly in RPC-C2A and MRC5 cells after 24h or 72h of exposure. The cytotoxic effects of Vitrebond and SuperEBA were statistically different ($P < 0.05$) in both cells lines and exposure periods.

Similar results were obtained after exposure to ProRoot MTA and MTA Angelus. Generally both materials showed

Table 1 Cytotoxicity on RPC-C2A cells of the tested materials after 24 and 72 h exposure expressed in percent of control

Material	Percent viable RPC-C2A cells	
	24 h	72 h
white MTA Angelus	92.49 ± 5.51 ^a	88.94 ± 6.81 ^A
white ProRoot MTA	91.20 ± 4.62 ^a	81.84 ± 6.01 ^A
Super EBA	52.37 ± 3.72 ^b	43.97 ± 3.29 ^B
Vitrebond	15.06 ± 2.88 ^c	10.43 ± 0.86 ^C

Data are shown as mean ± SD. Same letters in columns denote not statistically significant differences ($P > 0.05$).

Table 2 Cytotoxicity on MRC-5 cells of five materials at 24 and 72 h expressed in percent of control

Material	Percent viable MRC5 cells	
	24 h	72 h
white MTA Angelus	105.39 ± 6.06 ^a	90.26 ± 3.67 ^A
white ProRoot MTA	96.03 ± 4.19 ^a	92.15 ± 6.74 ^A
SuperEBA	63.25 ± 3.98 ^b	39.00 ± 2.51 ^B
Vitrebond	22.06 ± 5.12 ^c	19.43 ± 6.29 ^C

Data are shown as mean ± SD. Same letters in columns denote not statistically significant differences ($P > 0.05$).

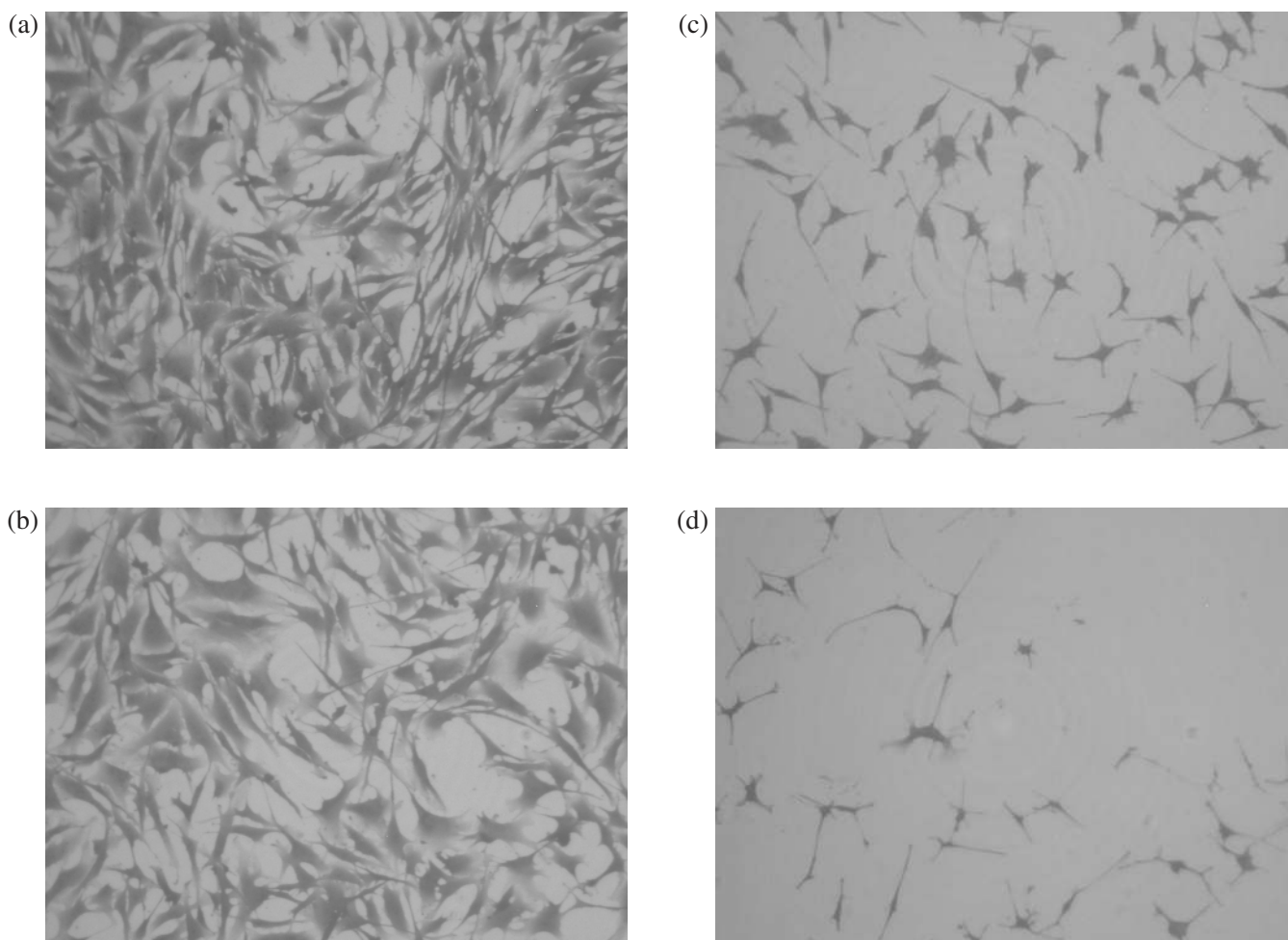


Fig. 1 Representative photos of cells (a) RPC-C2A exposed to white MTA Angelus, (b) RPC-C2A exposed to white ProRoot MTA, (c) MRC5 exposed to SuperEBA and (d) MRC5 exposed to Vitrebond ($\times 200$ magnification)

only slight inhibitory effect on cell viability. The effect of ProRoot MTA and MTA Angelus was statistically significant lower than the effect caused by exposure to Vitrebond and SuperEBA ($P < 0.05$).

Discussion

The results of the present study showed that MTA Angelus and ProRoot MTA were biocompatible when tested in rat pulp cells and in human lung fibroblasts. For the experimental setting, the cements were placed on the microporous membrane of an insert well that was floating over the culture medium in a multiwell plate. Thus, we evaluated the effects of the set materials on cell survival preventing physical interaction between the investigated material and the target cells (16) and compared the cell survival in the presence of the four tested cements.

RPC-C2A is a clonal cell line that has been established from the dental pulp tissue of the rat incisor by Kasugai et al. (17). The alkaline phosphatase of RPC-C2A cells is biochemically identical with that of the dental pulp and characterized by alkaline phosphatase (ALP) activity as a marker enzyme for cloning (17). These cells have high growth activity and are easily maintained in typical laboratory conditions. We have used this cell line in several cytotoxicity experiments (16,18,19). In the present experiment, we also used another cell line of human origin, the MRC5 cell line, in order to evaluate the differential sensitivity of the two lines. Indeed, the RPC-C2A cells were more sensitive than the MRC5 cells in all settings and percentage of cell survival was overall lower in the RPC-C2A cells.

The two brands of MTA were significantly less cytotoxic than SuperEBA and Vitrebond. These results are in agreement with other studies supporting the biocompatibility of MTA (16,20-22). No statistically significant differences were observed in the degree of the cytotoxic effect displayed by MTA-Angelus and ProRoot MTA. The two commercial brands of MTA have similar chemical composition although ProRoot MTA is reported to present slightly higher percentages of bismuth oxide than MTA Angelus (23,24). De Deus et al. (9) evaluated the cytotoxic effects of the two brands of MTA and Portland cement on human endothelial cells. No statistically significant difference was found among the materials tested, while the cytotoxic effect decreased gradually with time. In another study in which macrophages were exposed to MTA, the viability was greater than 97% at all examination periods and both ProRoot MTA and MTA Angelus exerted similar effect (8).

To the best of our knowledge, no published data exist about the cytotoxicity of MTA Angelus in dental pulp

cells. The results of the present study showed that MTA Angelus exhibited very slight cytotoxic effect against pulp cells (RPC-C2A). Our results support the good biological behavior of MTA Angelus and they are well correlated to the findings of two relevant studies (8,9) in which MTA Angelus is tested in endothelial cells and macrophages.

In this study, we tested the white formulations of MTA. White MTA has been introduced in clinical practice to overcome problems regarding coronal discoloration of teeth treated with gray MTA. Both white and gray types of MTA have similar cytotoxic effect (25) and when tested as pulpotomy agents, healing of the pulp and hard tissue bridge formation was observed (26).

SuperEBA cement consists of a powder containing zinc oxide (60-75%), fused quartz or alumina (20-35%) and hydrogenated resin (6%) and a liquid containing 63% ethoxybenzoic acid (EBA) and 37% eugenol. The EBA encourages the formation of a crystalline structure that improves the strength of the material. The zinc oxide-eugenol cements are generally inclined to cause inflammatory reactions in the tissues, mainly due to the presence of free eugenol. Several studies have shown the cytotoxic effect of SuperEBA that may be attributed to its eugenol content. Eugenol has been widely used as an antimicrobial and anti-inflammatory agent; however, previous *in vitro* and *in vivo* studies have demonstrated its toxic effects (27-32). It has been reported that eugenol inhibits cell migration, prostaglandin synthesis, cell respiration and mitochondrial activity (29-31). It also alters the cell membrane (29) and stimulates the neutrophils (30,32).

Vitrebond was the most potent material among those tested in the present study in all experimental settings. RMGICs have shown an increased cytotoxicity when tested in several studies. Souza et al. (33) evaluated the effect of three RMGICs applied on a culture of MDPC-23 cells or implanted into subcutaneous tissue of rats. The materials induced a noticeable inflammatory response when they came in direct contact with connective tissue and Vitrebond showed the highest cytotoxic effect. The addition of leachable resin components, such as 2-hydroxyethyl-methacrylate (HEMA), in RMGICs seems to be responsible for their cytotoxicity (34). HEMA is a very effective hydrophilic monomer that readily dissolves in water. It has been reported that Vitrebond releases a very high percentage of HEMA after immersion in distilled water, even when the material is light-cured (35). HEMA can suppress cell growth and proliferation (36), and can cause cell death by induction of apoptosis in cultured fibroblasts (37).

Under the present experimental conditions, ProRoot

MTA and MTA Angelus exerted similar, favorable effects on the mitochondrial activity of fibroblasts. Although the *in vitro* results are not directly transferable to *in vivo* conditions, the application of both cements in clinical practice is encouraged.

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