Cytotoxicity of MTA and Portland cement on human ECV 304 endothelial cells

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Abstract

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Aim To evaluate the cytotoxic effects of two brands of mineral trioxide aggregate (MTA) (Pro-Root MTA® and MTA Angelus®) and Portland cement (PC) on the human ECV 304 endothelial cell line.

Methodology Endothelial ECV 304 cells were incubated at 37 °C in an atmosphere of 95% air, 5% carbon dioxide and 100% humidity for 7 days and grown in F12 medium supplemented with 10% fetal bovine serum with 50 μg mL^{-1} of gentamicin sulphate. Effects of the materials on mitochondrial functions were measured by a colorimetric assay. At each experimental time interval (24, 48 and 72 h), a dimethyl-thiazol-diphenyl tetrazolium bromid assay was conducted to measure cell viability. All assays were repeated three times to ensure reproducibility. Results were expressed as average absorbance ($A_{570\,nm}$) ± SD and the data were analysed statistically by one-way analysis of variance and the Bonferroni post-test. A *P*-value <0.05 was considered statistically significant.

Results No statistically significant difference was shown between any of the experimental materials ($P > 0.05$).

Conclusions The two brands of MTA analysed, as well as the PC, initially showed a similar elevated cytotoxic effect that decreased gradually with time allowing the cell culture to become reestablished.

Keywords: cytotoxicity, mineral trioxide aggregate, portland cement.

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Introduction

Mineral trioxide aggregate (MTA) is an endodontic material that was developed at Loma Linda University (Loma Linda, CA, USA) in 1993 (Abdullah *et al.* 2002). The material was first used as a root end filling material (Torabinejad *et al.* 1993) but it has also been used as a viable alternative for various clinical applications, such as capping of pulp tissue, root end closure and for repairing furcal perforations (Torabinejad & Chivian 1999). Underlying these applications are the properties of MTA that include biocompatibility, good sealing ability and capability of promoting dental pulp and periradicular tissue regeneration (Abdullah *et al.* 2002). Perez *et al.* (2003) reported that MTA might be an ideal material because it consistently induced the regeneration of periodontal ligament tissues, the apposition of a cementum-like material and formation of bone. MTA has been reported to be biocompatible in many *in vivo* and *in vitro* studies. Koh *et al.* (1998) reported that MTA offered a biologically active substrate for bone and cells stimulating interleukin production and Mitchell *et al.* (1999) reported that MTA was biocompatible and suitable for clinical trials. Zhu *et al.* (2000) reported that osteoblasts have a favourable response to MTA.

Recently, the chemical, physical and biological properties of Portland cement (PC) were analysed. Wucherpfenning & Green (1999) reported that MTA and PC were similar macroscopically and microscopically when analysed by X-ray diffraction. Estrela *et al.* (2000) reported that PC contains the same principal chemical elements as MTA, except for bismuth oxide in

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MTA that increases the radiopacity of the material. Furthermore, osteoblast-like cells had similar growth and matrix formation when in contact with either MTA or PC (Wucherpfenning & Green 1999). MTA and PC have comparable antibacterial activity (Estrela *et al.* 2000). Saidon *et al.* (2003) reported that MTA and PC have similar properties but MTA is an expensive material whereas PC is an economical cement.

In vitro cytotoxic assays are simple, reproducible, cost-effective, relevant and suitable for evaluation of basic biological aspects relating to biocompatibility (Chang *et al.* 1998). In this kind of analyses, the dimethyl-thiazol-diphenyl tetrazolium bromid (MTT) assay has demonstrated some significant advantages such as: simplicity, rapidity and precision. Considering these facts and also that there has been very little data comparing the *in vitro* biocompatibility of these materials, the aim of this paper was to evaluate the cytotoxic effects of two brands of MTA [Pro-Root MTA® (Dentsply; Tulsa Dental, Tulsa, OK, USA) and MTA Angelus® (Curitiba, Paraná, Brazil)] and PC (MAUÁ CP32-TYPO II; Lafarge, Rio de Janeiro, Brazil) on the human ECV endothelial cell line by MTT assay. MTA Angelus is a competitive product of Pro-Root MTA®, made in Brazil.

Materials and methods

Sample preparation

The materials used in this study were Pro-Root MTA® (Dentsply; Tulsa Dental), MTA Angelus® (Curitiba) and PC (MAUÁ CP32-TYPO II; Lafarge). The Pro-Root and MTA Angelus were mixed according to the manufacturers' instructions. The PC was sterilized with ethylene oxide and mixed to a consistency similar to the other cements. Triplicate sample discs of the materials were fabricated in sterile cylindrical glass moulds that were 4 mm height and 3 mm in diameter. Excess flash was removed with a sterile scalpel. To prevent bacterial contamination, specimens were exposed to UV light for 30 min after fabrication and trimming.

Cell cultures

Human ECV 304 endothelial cells were obtained from spontaneously immortalized human umbilical chord veins. These were grown and maintained using procedures developed by Takahashi *et al.* (1990). The endothelial ECV 304 cells were incubated at 37 °C in an atmosphere of 95% air, 5% carbon dioxide and 100% humidity for 7 days and grown in F12 medium supplemented with 10% fetal bovine serum (FBS; Hyclone, LLogan, UT, USA) with 50 μg mL^{-1} of gentamicin sulphate (Sigma Chemical Co., St Louis, MO, USA). ECV 304 cells were collected by washing with serum free α-MEM (Sigma Chemical Co.) before treatment with 5 mL trypsin (0.1%)/1 mL EDTA (0.01%) (Sigma Chemical Co.) solution in phosphate-buffered saline for 7–10 min. Cells from the fourth collection were plated in a 96-well plate at a density of 1.0×10^5 cells per well and allowed to attach for 24 h to an α-MEM plus supplements. Medium with supplements alone provided negative controls.

Metabolic assay

Effects of the materials on mitochondrial function were measured by a colorimetric assay as described by Mosmann (1983). Upon incubation with viable cells, the tetrazolium ring of MTT (pale yellow) is cleaved by cellular deydrogenases enzymes to convert the yellow water-soluble tetrazolium salt MTT into dark blue formazan crystals. MTT solution (0.5 mg mL^{-1} per well) was added to each plate and they were incubated to be solubilized with dimethylsulphoxide (200 $\frac{1}{4}$) and the absorbance determined at $A_{570\ nm}$ using an ELISA plate reader (Thermomax Microplate Reader; Molecular Devices, Santa Monica, CA, USA). At each experimental time period (24, 48 and 72 h), an MTT assay was conducted to measure cell viability. Optical microscopic analyses of the morphology of the untreated control and the cement-treated cell cultures were carried out in the three experimental periods.

Statistical analysis

All assays were repeated three times to guarantee reproducibility. Results were expressed as average absorbance ($A_{570\backslash,nm}$) ± SD and a multiple linear regression model (SPSS/PC + Statistics 4.0 software; SPDD International BV, Gorinchem, the Netherlands) were used. The significance of the difference between the control and experimental groups was statistically analysed by one-way analysis of variance and the Bonferroni post-test. A P-value <0.05 was considered statistically significant.

Results

A confluent cell culture was observed in a control group maintained for the whole time of the experiment (72 h) (Fig. 1). For the first MTT assay (24 h), a

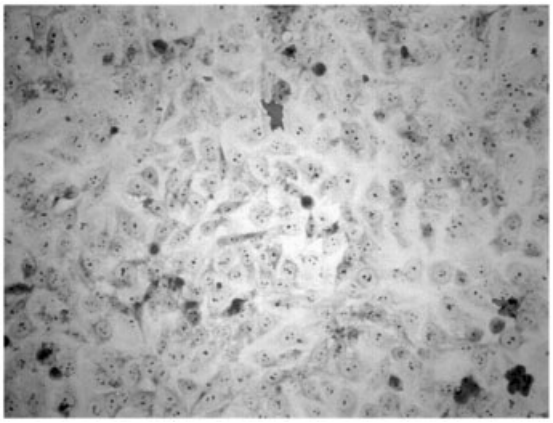

Figure 1 Human endothelial cells (ECV 304) in control culture maintain a confluent monolayer after 72 h (×50).

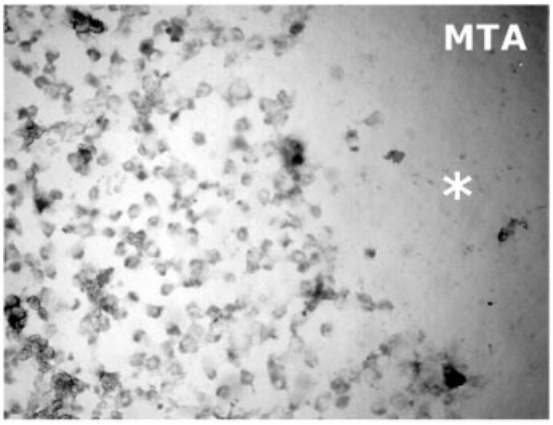

Figure 2 Human endothelial cells (ECV 304) exposed to Pro-Root mineral trioxide aggregate for 24 h. Cells with morphological alterations were observed (×50). *Region with high cytotoxicity effect with general cellular death.

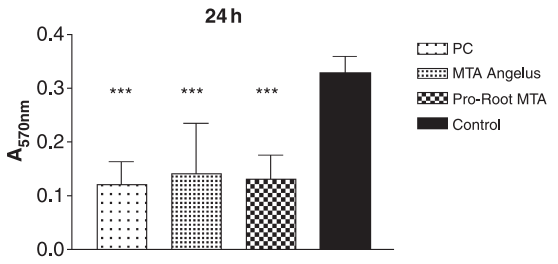

Figure 3 Effect of elutes of Pro-Root mineral trioxide aggregate (MTA), MTA Angelus, Portland cement and control group at 24 h on human endothelial cells (ECV 304) by dimethyl-thiazol-diphenyl tetrazolium bromid assay. Each bar represents average absorbance ($A_{570\ nm}$) ± SD. ***Significant differences from control values for $P < 0.001$.

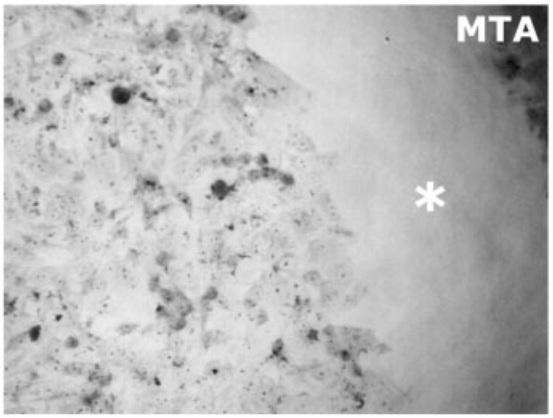

Figure 4 Human endothelial cells (ECV 304) exposed to Pro-Root mineral trioxide aggregate for 72 h. Cells with lower morphology alteration were observed (×50). *The high initial cytotoxic effects are decreased gradually over the experimental time period allowing the cell culture to repair.

statistically significant difference was found ($P < 0.001$) between the experimental materials and the control group. In this experimental moment, all cements inhibited cell viability. In all experimental groups, changes in cell morphology in human endothelial cells were observed near the material sample at the end of the first 24 h (Fig. 2) but no statistically significant difference was found between any experimental materials. These results were expressed as means of the absorbance ($A_{570\ nm}$) ± SD of each material and the control group in Fig. 3.

For the second and the third MTT assays (48 and 72 h), all the three materials exhibited a similar slight inhibitory effect on cell viability (Fig. 4); no statistically significant difference was found between the experimental materials, the control group, nor between any of the experimental materials. These results were expressed as the average of the absorbance ($A_{570\ nm}$) ± SD of each material and the control group in Figs 5 and 6.

Discussion

Ideally, endodontic materials should be biocompatible and have satisfactory physicochemical properties. Although many reports have investigated the physicochemical proprieties of MTA (Aquilina 1999, Wucherpfenning & Green 1999), only a few compare MTA with PC in terms of biocompatibility. The toxic

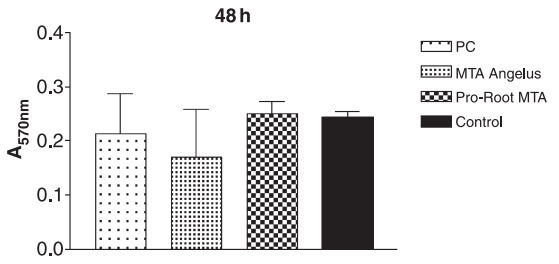

Figure 5 Effect of elutes of Pro-Root mineral trioxide aggregate (MTA), MTA Angelus, Portland cement and control group at 48 h on human endothelial cells (ECV 304) by dimethyl-thiazol-diphenyl tetrazolium bromid assay. Each bar represents average absorbance ($A_{570\ nm}$) ± SD. No significant differences between any of the groups from $P > 0.05$.

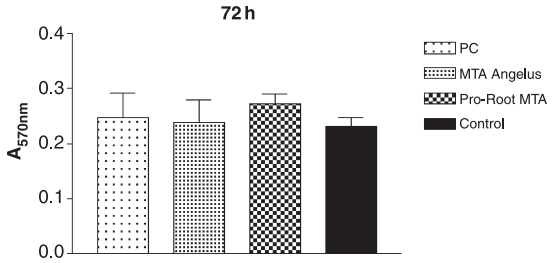

Figure 6 Effect of elutes of Pro-Root mineral trioxide aggregate (MTA), MTA Angelus, Portland cement and control group at 72 h on human endothelial cells (ECV 304) by dimethyl-thiazol-diphenyl tetrazolium bromid assay. Each bar represents average absorbance ($A_{570\ nm}$) ± SD. No significant differences between any of the groups from $P > 0.05$.

effects of materials used for endodontic therapy are of particular concern, because damage or irritation could cause degeneration of the periapical tissue and delayed wound healing.

In vivo tests, such as implantation and usage tests, have an advantage in that they allow complex interactions between the host and the material to be examined. *In vitro* tests such as cell culture enable experimental factors and variables to be controlled, which often is a significant problem when performing experiments *in vivo* (Spångberg 1969). These *in vitro* model assays are increasingly being used for initial screening of new dental materials intended for use in humans. The decision to use a particular test system should be based on its consonance with the chemical nature of the material, this being the permeability of cell membranes, making a permeability assay less apt to determine cytotoxicity in a valid manner (Keiser *et al.* 2000). As MTA is a hydrophilic material likely to release ionic components, it would be more fitting to interfere with intracellular enzyme activities than influence membrane permeabilities (Scweikl *et al.* 1996, Keiser *et al.* 2000). In this project, the cell viability was determined qualitatively through the optical microscopic comparison of the morphology of an untreated control and the cement-treated cell cultures, and quantitatively by the MTT assay based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble tetrazolium salt MTT into dark blue formazan crystals. The advantages of this method are its simplicity, rapidity and precision. In addition, it does not require radioisotopes (Huang *et al.* 2002). The human endothelial cell line (EVC-304), which has also been used in previous apoptosis studies (Escargueil-Blanc *et al.* 1998) was chosen to simulate the *in vivo* situation.

Previous research has demonstrated that MTA and PC provide good biocompatibility (Mitchell *et al.* 1999, Keiser *et al.* 2000, Holland *et al.* 2001a,b, Abdullah *et al.* 2002). Abdullah *et al.* (2002) reported that accelerated PC supported the proliferation of SaOS-2 osteosarcoma line cells *in vitro* and actively stimulated a biological response in these cells through the production of cytokines and a bone-specific protein. These authors affirmed that it also formed a significant step in the development of PC as a restorative material. Holland *et al.* (2001a), studying the response of dental pulp of dog after pulpotomy and subsequently protection of the remaining tissue with MTA and PC, reported that both materials had similar chemical formulations, except for bismuth oxide in MTA and the results were the same for MTA and PC. The results of biological research with MTA and PC are supported by Holland *et al.* (2001b) who reported a similar action of MTA and PC. Both materials have calcium oxide that forms calcium hydroxide when mixed with water. The reaction of calcium hydroxide and the carbon dioxide from pulp tissue produces calcite crystals (Holland *et al.* 2001b). Seux *et al.* (1991) concluded that their findings strongly support the role of calcite crystals and fibronectin as an initiating step in the formation of a hard tissue barrier.

The present results are compatible with these described previously. Statistical analyses of the data of the MTT assay showed no significant difference between the three cements in all experimental periods (24, 48 and 72 h) (Figs 3, 5 and 6). A significant difference was found between the experimental materials and the control group ($P < 0.001$) in the first MTT

assay. Therefore, all cements significantly inhibited cell viability. At this time, all experimental groups showed changes in cell morphology near the material samples (Fig. 2). This initial cytotoxic effect might occur because of the high surface pH of the cements that cause denaturation of adjacent cells and medium proteins. The results are closer to those of Saidon *et al.* (2003) who reported that as the cement set pH decreased the cell injuries subsided. For the second and the third MTT assays (48 and 72 h), all three cements exhibited a similar slight inhibitory effect on cell viability (Fig. 4) and no statistically significant difference between the experimental materials and the control group, nor between any of the experimental materials. For the second and the third MTT assays, changes in cell morphology were found in low number comparable with the first 24 h. The high initial cytotoxic effects decreased gradually over the experimental time period allowing the cell culture to repair. These results also support those described previously by others (Estrela *et al.* 2000, Holland *et al.* 2001a,b, Abdullah *et al.* 2002, Saidon *et al.* 2003) who suggested that MTA and PC share similar biological proprieties.

Conclusion

Within the parameters of this *in vitro* evaluation, the authors arrived at the following conclusions:

1. The two brands of MTA analysed, as well as the PC, initially showed an elevated cytotoxic effect that decreased gradually with time allowing the cell culture to repair.

2. The cell reaction patterns were similar for Pro-Root MTA, MTA Angelus and PC in all experimental time periods.

3. The positive biological results of this research are encouraging for the use of PC as an endodontic restorative material, but more studies are necessary before warranting unlimited clinical use.

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