Ex vivo biocompatibility tests of regular and white forms of mineral trioxide aggregate

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Abstract

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Aim To examine the genotoxicity and cytotoxicity of regular and white mineral trioxide aggregate (MTA) *ex vivo* by the single-cell gel (comet) assay and trypan blue exclusion test, respectively.

Methodology Aliquots of 1×10^4 Chinese hamster ovary cells were incubated at 37 °C for 3 h with grey and white forms of MTA at final concentrations ranging from 1 to 1000 μg mL^{-1}. The negative control group was treated with vehicle control phosphate buffer solution for 3 h at 37 °C and the positive control group was treated with methyl metasulfonate (at 1 μg mL^{-1}) for 1 h at 37 °C. After incubation, the cells were centrifuged at 180 *g* for 5 min and washed twice with fresh medium and resuspended with fresh medium. Each individual treatment was repeated three times consecutively to ensure reproducibility. Parameters from single-cell gel (comet) and cytotoxicity assays were assessed by the Kruskal–Wallis nonparametric test.

Results Neither compounds produced genotoxic effects with respect to the single-cell gel (comet) assay in all concentrations evaluated. In the same way, the dose–response relationships of all compounds tested at concentrations ranging from 1 to 1000 μg mL^{-1} on cell viability assessed by the trypan blue assay displayed no statistically significant differences ($P > 0.05$) for either endodontic material.

Conclusions Regular (grey) and white MTA are not genotoxins and do not induce cellular death.

Keywords: Chinese hamster ovary cells, cytotoxicity, genotoxicity, mineral trioxide aggregate.

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Introduction

Properties of a good apical root-end-filling material include the ability to adhere and seal the root canal system (Lamb *et al.* 2003). The material should also be easy to manipulate, be radiopaque, dimensionally stable, nonabsorbable, biocompatible with the periradicular tissues and nontoxic (Gartner & Dom 1992). In the 1990s, mineral trioxide aggregate (MTA) (which is grey in colour) was developed as a root-end-filling material. A number of biocompatibility studies have been conducted either *in vitro* or *in vivo*, which have shown that MTA has good sealing ability and tissue healing properties (Torabinejad *et al.* 1995a,b,c, Koh *et al.* 1997, 1998, Holland *et al.* 1999, Mitchell *et al.* 1999, Menezes *et al.* 2004). Recently, a new tooth-coloured form of MTA has been developed for use (Perez *et al.* 2003), but comparatively little information is available on the biocompatibility of these compounds (Camilleri *et al.* 2004).

Genotoxicity tests can be defined as *in vitro* and *in vivo* tests designed to detect compounds which induce genetic damage including DNA damage, gene mutation, chromosomal breakage, altered DNA repair capacity and cellular transformation. Genotoxicity assays have gained widespread acceptance as an important and useful indicator of carcinogenicity

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(Auletta & Ashby 1988). For this reason, genotoxicity data are needed for the comprehensive risk assessment of MTA (Kettering & Torabinejad 1995).

Over the past decade, the single-cell gel (comet) assay in alkaline version was developed as a rapid, simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells (Tice *et al.* 2000). The basic principle of the single-cell gel (comet) assay is the migration of DNA fragments in an agarose matrix under electrophoresis. When viewed under a microscope, cells have the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating towards the anode (Olive *et al.* 1990). Recent studies have demonstrated that the single-cell gel (comet) assay is a suitable tool to investigate genotoxicity of compounds used in dental practice (Ribeiro *et al.* 2004a, 2005).

Therefore, the purpose of this study was to investigate whether two commercial forms of MTA can induce DNA breakage in Chinese hamster ovary (CHO) cells by the single-cell gel (comet) assay. To monitor cytotoxic effects, the trypan blue exclusion test was applied.

Material and methods

Cell culture

Chinese hamster ovary K-1 cells were grown to confluence in 75-cm^2 culture flasks (Corning, New York, NY, USA) using Ham's F-10 medium (Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% foetal calf serum and 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Invitrogen Corporation) incubated in a 95% air, 5% CO_2 atmosphere at 37 °C. Cells were cultured for 5 days prior to treatment with test substances. Confluent cells were detached with 0.15% trypsin (Invitrogen Corporation) for 5 min after that, 2 mL complete medium was added and cells were centrifuged at 180 *g* for 5 min. Cell suspensions were counted using a Neubauer® chamber (Herka, Germany) and seeded in 96-well microtitre plates (Corning) at a density of 1×10^4 cells per well (at a concentration of 1×10^6 mL^{-1}). All the procedures in this study were compatible with those described by the Ethics Committee of Botucatu Medical School, UNESP, SP, Brazil.

Treatment of cells

The materials used were MTA (regular and white) (Angelus; Soluções Odontológicas, Londrina, PR, Brazil). All materials tested were prepared in increasing final concentrations ranging from 1 to 1000 μg mL^{-1} and exposed for 3 h at 37 °C. The negative control group was treated with vehicle control phosphate buffer solution (PBS) for 3 h at 37 °C and the positive control group was treated with methyl metasulfonate (MMS at 1 μg mL^{-1}; Sigma Aldrich, St Louis, MO, USA) for 1 h at 37 °C. After incubating, the cells were centrifuged at 180 *g* for 5 min and washed twice with fresh medium and resuspended with fresh medium. Each individual treatment was repeated three times consecutively to ensure reproducibility.

Cytotoxicity assay

Cytotoxicity testing was performed using Trypan blue staining after the treatment (McKelvey-Martin *et al.* 1993). In brief, a freshly prepared solution of 10 μL Trypan blue (0.05%) in distilled water was mixed with 10 μL of each cellular suspension for 5 min, spread onto a microscope slide and covered with a coverslip. Nonviable cells appear blue stained. At least 200 cells were counted per treatment.

Single-cell gel (comet) assay

The protocol used for single-cell gel (comet) assay followed the guidelines purposed by Tice *et al.* (2000). Briefly, a volume of 10 μL of treated or control cells ($\sim 1 \times 10^4$ cells) were added to 120 μL of 0.5% low-melting point agarose at 37 °C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed and slides immersed in lysis solution [2.5 M NaCl, 100 mM EDTA (Merck, Darmstadt, Germany); 10 mM Tris–HCl buffer pH 10 (Sigma Aldrich, St Louis, MO, USA); 1% sodium sarcosinate (Sigma); with 1% Triton X-100 (Sigma); and 10% DMSO (Merck)] for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer [0.3 mM NaOH (Merck); and 1 mM EDTA (Merck); pH > 13] for 20 min and electrophoresed for another 20 min, at 25 V (0.86 V cm^{-1}) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris–HCl (pH = 7.5), fixed in absolute ethanol and stored at room temperature until analysis. In order to minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

Comet capture and analysis

A total of 50 randomly captured comets from each slide (Hartmann *et al.* 2003) were examined blindly at 400× magnification using a fluorescence microscope (Olympus, Orangeburg, NY, USA) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments, Haverhill, UK). A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To quantify the DNA damage, tail moment was evaluated. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. The comet tail moment is positively correlated with the level of DNA breakage in a cell. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

Statistical methods

Data of three independent repeats from the comet assay and the cytotoxicity were assessed by the Kruskal–Wallis nonparametric test, using SigmaStat software, version 1.0 (Jadel Scientific, Chicago, IL, USA). The level of statistical significance was set at 5%.

Results

Data were expressed as mean ± standard deviation from three independent experiments for grey and white MTA. The cytotoxicity of MTA (grey and white) was measured in CHO cells through trypan blue assay in range-finding experiment prior to the determination of chemically induced genotoxicity. The dose–response relationships of all compounds tested at concentrations ranging from 1 to 1000 μg mL^{-1} on cell viability assessed by trypan blue assay are shown in Fig. 1. No significant statistically differences ($P > 0.05$) of viable cells were found for both endodontic materials tested and in all concentrations tested.

The results of the alkaline single-cell gel (comet) assay were displayed in Table 1. DNA strand breaks were represented by the mean tail moment for 50 comets/sample. As seen in Table 1, neither material induced DNA strand breaks in all concentrations tested. For comparison, the comet assay was able to detect the significant increase in tail moment of positive control (MMS) with respect to negative control at high levels of cellular viability.

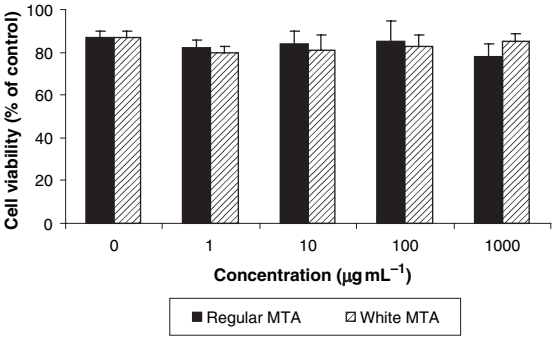

Figure 1 Effects of serial concentrations of mineral trioxide aggregate on trypan blue exclusion test. Results are expressed as the mean percentage of control (mean ± SD of three independent repeats).

Table 1 Mean ± SD of DNA damage (tail moment) in Chinese hamster ovary cells exposed to regular and white mineral trioxide aggregate (MTA)

	MTA	
Concentration (μg mL^{-1})	Regular	White
1000	0.84 ± 0.40[a]	0.64 ± 0.29
100	0.67 ± 0.17	0.67 ± 0.30
10	0.75 ± 0.32	0.48 ± 0.20
1	0.42 ± 0.23	0.76 ± 0.25
Negative control[b]	0.58 ± 0.16	0.58 ± 0.16
Positive control[c]	4.54 ± 0.74*	4.54 ± 0.74*

[a]Data of three independent repeats.
[b]Phosphate buffer solution (pH 7.4).
[c]MMS at 1 μg mL^{-1}.
*$P < 0.05$ when compared with negative control.

Discussion

The aim of this study was to evaluate the genotoxic and cytotoxic damage that MTA induced on CHO cells *ex vivo*. The choice of cell line and use of cells in continuous culture permits an accurate evaluation of the changes, independently from factors such as age, metabolic and hormonal states of the donor, which may influence the cell in primary culture. However, there are drawbacks associated with cell lines; for example, the chromosomal material has usually undergone extensive rearrangement, in which some important genes may be mutated. Even so, CHO cells have a small number of relatively large chromosomes; they

grow fast and reproducible results can be obtained from the same cell source if cells are stored in a frozen state.

The alkaline version of the single-cell gel (comet) assay used in the present study is sensitive for a wide variety of DNA lesions. Among them are single- and double strand breaks, oxidative DNA base damage, alkali-labile sites including abasic and incomplete repair sites, and DNA–DNA/DNA–protein/DNA–drug cross-linking in any eukaryotic cell (Tice *et al.* 2000). Tail moment is a virtual measure calculated by the computerized image analysis system considering both the length of DNA migration in the comet tail and the tail intensity. This parameter is one of the best indices of induced DNA damage among the various parameters calculated by this method (Hartmann *et al.* 2003). The results of this study pointed out that the alkaline single-cell gel (comet) assay in the experimental conditions used, failed to detect the presence of DNA damage after treatment by two forms of MTA in all concentrations tested. These findings confirmed and extended the data already published showing the good biocompatibility of MTA (Holland *et al.* 1999, Menezes *et al.* 2004). However, the single-cell gel (comet) assay does not necessarily predict the mutagenic potential of the test compound. One possible explanation for the absence of a close relationship to mutagenesis is that the effects seen in the single-cell gel (comet) assay for some substances mainly occur as a consequence of an error free DNA repair process (Speit *et al.* 1996).

The trypan blue exclusion test can be used to indicate cytotoxicity, where dead cells take up the blue stain of trypan blue, whereas the live cells have yellow nuclei. Although it does not give any detailed information other than membrane stability of the cell, it is a standardized and accepted test to monitor possible cytotoxic effects (McKelvey-Martin *et al.* 1993). Cytotoxicity data on CHO cultures demonstrated that in all concentrations assessed, both regular or white MTA were unable to produce cellular death. These results are in line with previous studies reporting that MTA was found to be nontoxic *in vitro* (Torabinejad *et al.* 1995b, Osório *et al.* 1998, Keiser *et al.* 2000).

In the present study, as well as in previous investigations using the single-cell gel (comet) assay, comets without clearly identifiable heads during the image analysis were excluded. Although it should be emphasized that it is still not completely understood what these 'clouds' actually represent, this type of comet was excluded on the basis of the assumption that these cells represent dead cells, resulting from putative cytotoxic effects of root-end-filling materials rather than primary DNA-damage following a direct interaction between DNA and a genotoxic agent (Ribeiro *et al.* 2004b).

Conclusions

Using the single-cell gel (comet) assay to evaluate DNA damage and cellular death by trypan blue staining, it was concluded that grey and white MTA do not induce noxious activities. Because DNA damage and cellular death are important events for carcinogenesis; this study represents an important contribution to the correct evaluation of the potential risks associated with exposure to dental materials. Furthermore, the results presented here might be an additional argument to support the use of MTA in endodontic practice.

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