

Biocompatibility In Vitro Tests of Mineral Trioxide Aggregate and Regular and White Portland Cements

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Abstract

Mineral trioxide aggregate (MTA) and Portland cement are being used in dentistry as root end-filling materials. However, biocompatibility data concerning genotoxicity and cytotoxicity are needed for complete risk assessment of these compounds. In the present study, genotoxic and cytotoxic effects of MTA and Portland cements were evaluated in vitro using the alkaline single cell gel (comet) assay and trypan blue exclusion test, respectively, on mouse lymphoma cells. The results demonstrated that the single cell gel (comet) assay failed to detect DNA damage after a treatment of cells by MTA and Portland cements for concentrations up to 1000 $\mu\text{g/ml}$. Similarly, results showed that none of the compounds tested were cytotoxic. Taken together, these results seem to indicate that MTA and Portland cements are not genotoxins and do not induce cellular death.

Key Words

Mineral trioxide aggregate, Portland cement, comet assay, genotoxicity

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Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application (1). This means that the tissue of the patient that comes into contact with the materials does not suffer from any toxic, irritating, inflammatory, allergic, genotoxic, or carcinogenic action (2, 3).

Over the past decade, a new material, mineral trioxide aggregate (MTA) was developed as a root-end-filling material (4). Herein, the biocompatibility of MTA has been investigated in many studies through bioassays in vivo and in vitro (5–19). Recently, studies have compared MTA with Portland cement and the findings suggest that they seem almost identical macroscopically, microscopically, and by X-ray diffraction analysis (20). Other study affirms that Portland cements contain the same chemical elements as MTA (21). This suggests that Portland cement has the potential to be used as a less expensive root-end-filling material in dental practice (22).

Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds that 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 (23). For this reason, genotoxicity data are needed for complete risk assessment of MTA and Portland cements, particularly because there are no previous reports.

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 (24). 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. Previous studies conducted by our group have proved that single cell gel (comet) assay is a suitable experimental model to test genotoxicity (25–29).

Therefore, the aim of the present study was to evaluate in vitro genotoxic effects of MTA and Portland cements in mouse lymphoma cells by the single cell gel (comet) assay. Mouse lymphoma cells were chosen to study the genotoxicity because the mechanism of DNA damage induced in these cells has been well documented. To monitor cytotoxic effects, trypan blue exclusion test was applied.

Materials and Methods

Cell Culture

L5178Y mouse lymphoma cells were cultivated in suspension in RPMI 1640 glutamax medium (Life Sciences, St. Petersburg, FL) supplemented with 10% heat-inactivated horse serum and penicillin/streptomycin (Life Technologies, Rockville, MD) at 37°C with 5% CO₂ according to Rothfuss et al. (30). Mouse lymphoma cells were first defrosted and subsequently subcultivated three times before performing the experiment. Cell suspension was counted using a Neubauer chamber and seeded in 96-well microtitre plated (Corning Glass, Corning, NY) at a density of 1×10^4 cells per well (at a concentration of $1 \times 10^6/\text{ml}$). All the procedures in this study concern ethical conducts described by Committee of Botucatu Medical School, SP, Brazil.

Treatment

The materials used were MTA Angelus (Angelus Soluções Odontológicas, Londrina, Brazil), Portland cement (Votorantim-Cimentos, São Paulo, Brazil) and white Portland cement (Votorantim-Cimentos, São Paulo, Brazil). All materials tested were prepared in increasing final concentrations ranging from 1 to 1000 µg/ml. The negative control group was treated with vehicle control (PBS) and the positive control group was treated with methyl metasulfonate (MMS at 10 µg/ml, Sigma Aldrich, St. Louis, MO). After incubating for 3 h at 37°C, the cells were centrifuged at 1000 rpm (180 G) for 5 min and washed three times with fresh medium and resuspended with fresh medium. Each individual treatment was repeated three times consecutively to ensure reproducibility.

Cytotoxicity Assay

Cytotoxicity was performed using Trypan blue staining after the treatment (31). In brief, a freshly prepared solution of 10 µl Tripan blue (0.05%) in distilled water was mixed to 10 µl of each cellular suspension during 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.

Genotoxicity Assay

The protocol used for single cell gel (comet) assay followed the guidelines purposed by Tice et al. (24). Briefly, a volume of 10 µl of cells (~1 × 10⁴ cells) of each treatment was added to 120 µl of 0.5% low-melting point agarose at 37°C, layered onto a precoated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and slides immersed to lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris-HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for about 1 h. Before electrophoresis, the slides were left in alkaline buffer (pH >13) for 20 min and electrohored for another 20 min, at 25V (0.86 V/cm) 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 blindly in a fluorescence microscope at 400× magnification. An automatized analysis system (Comet Assay II, Perceptive Instruments, Haverhill, Suffolk, UK) was used to determine DNA damage. Tail moment (product of tail DNA/total DNA by the center of gravity) was considered to estimate DNA damage from 50 cells per treatment (32). To minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

Statistical Methods

Parameters from the comet assay and the cytotoxicity were assessed by the Kruskal-Wallis nonparametric test, using SigmaStat software, version 1.0 (Jadel Scientific, Rafael, CA). The level of statistical significance was set at 5%.

Results

The toxicity of the different test compounds to mouse lymphoma cells was measured for concentrations ranging from 1 to 1000 µg/mL. In these conditions, cell mortality was found <15%, including the cell cultures exposed to the highest concentration of MTA and regular and white Portland cements. The dose-response relationships of all compounds tested at concentrations ranging from 0 to 1000 µg/mL on cell viability assessed by trypan blue assay are shown in Fig. 1.

The results of the alkaline single cell gel (comet) assay were displayed in Table 1. No primary DNA damage was observed after cell exposure to MTA and Portland cements. In all treatment conditions, none of the three compounds increased cell mortality. For comparison,

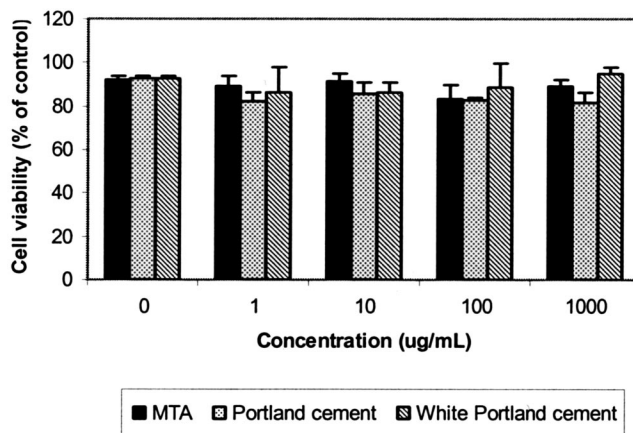


Figure 1. Effects of serial concentrations of MTA and Portland cements on trypan blue exclusion test. Results are expressed as the mean percentage of control (mean ± SD).

TABLE 1. Mean ± SD of DNA damage (tail moment) in mouse lymphoma cells exposed to MTA and Portland cements

Concentration (µg/ml)	MTA	Portland Cement	White Portland Cement
1000	0.85 ± 0.33	0.67 ± 0.49	0.72 ± 0.64
100	0.90 ± 0.50	0.89 ± 0.23	0.94 ± 0.24
10	0.89 ± 0.50	0.54 ± 0.41	0.89 ± 0.51
1	0.59 ± 0.20	0.89 ± 0.21	0.75 ± 0.22
Negative control ^a	0.82 ± 0.30	0.82 ± 0.30	0.82 ± 0.30
Positive control ^b	5.18 ± 0.84*	5.18 ± 0.84*	5.18 ± 0.84*

^aPhosphate buffer solution (ph 7.4).

^bMMS at 10 µg/ml.

*p < 0.05 when compared to negative control.

the comet assay was able to detect the significant increase in tail moment of positive control (MMS) with respect to negative control.

Discussion

In this study, the cytotoxic and genotoxic potential of MTA and Portland cements were investigated in vitro using the trypan blue exclusion test and alkaline single cell gel (comet) assay, respectively. In vitro studies are simple, inexpensive to perform, provide a significant amount of information, can be conducted under controlled conditions, and may elucidate the mechanisms of cellular toxicity (33). The results obtained from in vitro assays might be indicative of the effects observed in vivo. The trypan blue exclusion test can be used to indicate cytotoxicity, where dead cells take up the blue stain of trypan blue, while the live cell have yellow nuclei. The results presented in this study pointed out that MTA did not produce cellular death using the trypan blue assay. These findings confirmed and extended the data already published showing an absence of cytotoxic activity of MTA (19, 34–36). Similarly, no measurable cytotoxicity was observed for both regular and white Portland cements. Because the applied methods have been widely used for the detection and characterization of possible hazardous xenobiotics, a specific range of genotoxic effects is commonly agreed to be present (24). Therefore, common agreement exists that for the overall estimation of a genotoxic potential, a battery of tests should be applied (28). The single cell gel (comet) assay is a sensitive method for the detection of DNA damage induced by genotoxic compounds in individual cells. The alkaline version, used in this study, is able to detect a variety of DNA lesions including DNA strand breaks, alkali labile lesions, incomplete

repair sites, and abasic sites (32). It shows clear advantages concerning the applicability of almost all kind of cell types. Taking into account the lack of data currently available, the assessment of the potential genotoxicity of MTA and Portland cements by the comet assay remained justified.

The results of this study showed that the alkaline single cell gel (comet) assay, in the experimental conditions used, failed to detect the presence of DNA damage after a treatment by MTA up to 1000 $\mu\text{g/ml}$. This absence of primary DNA damage was also found for two Portland cements. MTA contains the same chemical elements as Portland cements (21); both gray and white Portland cements are manufactured from similar raw materials except that a fluxing agent is used for production of the white version to remove the ferrite phase during the clinkering process (37). Probably, this explains the same results obtained from genotoxicity between MTA and Portland cements. Nevertheless, for the more detailed judgment on the genotoxic potential of the MTA and Portland cements, further investigation is needed.

In the present study, as well as in all of our previous investigations using the single cell gel (comet) assay, we have always excluded comets without clearly identifiable heads during the image analysis. 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 after a direct interaction between DNA and a genotoxic agent (38). Here, no relationship was found between frequency of clouds and endodontic materials tested.

In conclusion, the results clearly indicate that MTA and Portland cements had no cytotoxic effects in mouse lymphoma cells. In the same way, all root-end-filling materials tested did not induce DNA damage as depicted by the single cell gel (comet) assay. The results presented here might be an additional argument to support the use of MTA and Portland cements in dental practice.

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