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

Daniel Araki Ribeiro, PhD, Marco Antonio Hungaro Duarte, PhD,† Mariza Akemi Matsumoto, PhD,† Mariangela Esther Alencar Marques, PhD,* and Daisy Maria Favero Salvadori, PhD**

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 μg/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

From the Center for Genotoxins and Carcinogens Evaluation (TOXICAN), Department of Pathology, Botucatu Medical School—UNESP; and Department of Dental Clinics, University of Sagrado Coração—USC.

Address request for reprints to Dr. Daniel Araki Ribeiro, PhD, TOXICAN, Departamento de Patologia, Faculdade de Medicina de Botucatu—UNESP, Distrito de Rubião Jr s/n Botucatu, SP Brazil 18618-000. E-mail address: ak92@hotmail.com.

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. Petersburgh, FL) supplemented with 10% heat-inactivated horse serum and penicillin/streptomycin (Life Technologies, Rockville, MD) at 37°C with 5% CO_2 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 1×10^6/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 ($\sim 1 \times 10^4$ 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 NaCI, 100 mM EDTA, 10 mM Tris-HCI 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 electrohoresed for another 20 min, at 25V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCI (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, Sufolk, 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, the comet assay was able to detect the significant increase in tail moment of positive control (MMS) with respect to negative control.

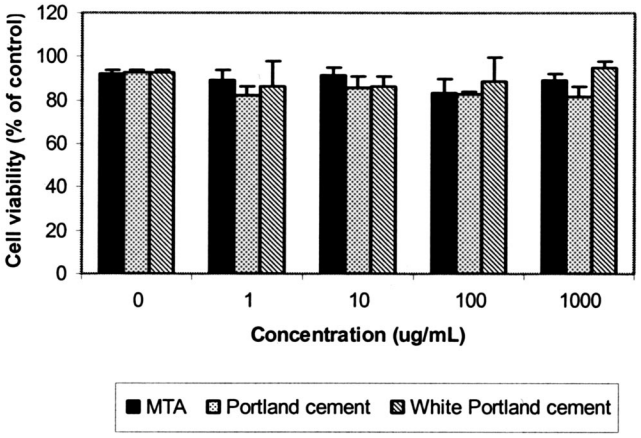

Figure 1. Effects of serial concentrations of MTA and Portland cements on trypan blue exclusion test. Results are expressed as the mean percentage of control (mean ± SD).

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*

[a]Phosphate buffer solution (ph 7.4).

[b]MMS at 10 μg/ml.

*$p < 0.05$ when compared 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 μg/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.

Acknowledgments

CNPq (Conselho de Desenvolvimento Cientifico e Tecnologico), FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and TOXICAN (Nucleo de Avaliação Toxicogenética e Cancerigena) supported this study.

References

1. Willians DF. Definitions in biomaterials. Proceedings of a Consensus Conference of the European Society for Biomaterials. England co. 4, New York: Elsevier, 1986.
2. Lin Sun Z, Wataha JC, Hanks CT. Effects of metal ions on osteoblast-like cell metabolism and differentiation. J Biomed Mater Res 1997;34:29–37.
3. Valey JW, Simonian PT, Conrad EU. Carcinogenicity and metallic implants. Am J Orthod 1995;24:319–24.
4. Camilleri J, Montesin FE, Papaioannou S, McDonald F, Pitt Ford TR. Biocompatibility of two commercial forms of mineral trioxide aggregate. Int Endod J 2004;37:699–704.
5. Apaydin ES, Shabahang S, Torabinejad M. Hard-tissue healing after application of fresh or set MTA as root-end-filling material. J Endod 2004;30:21–4.
6. Balto HA. Attachment and morphological behavior of human periodontal ligament fibroblasts to mineral trioxide aggregate: a scanning electron microscope study. J Endod 2004;30:25–9.
7. Ferris DM, Baumgartner JC. Perforation repair comparing two types of mineral trioxide aggregate. J Endod 2004;30:422–4.
8. Hayashi M, Shimizu A, Ebisu S. MTA for obturation of mandibular central incisors with open apices: case report. J Endod 2004;30:120–2.
9. Main C, Mirzayan N, Shabahang S, Torabinejad M. Repair of root perforations using mineral trioxide aggregate: a long-term study. J Endod 2004;30:80–3.
10. Yaltirik M, Ozbas H, Bilgic B, Issever H. Reactions of connective tissue to mineral trioxide aggregate and amalgam. J Endod 2004;30:95–9.
11. Al-Nahzan S, Al-Judai A. Evaluation of antifungical activity of mineral trioxide aggregate. J Endod 2003;29:826–7.
12. Fridland M, Rosado R. Mineral trioxide aggregate (MTA) solubility and porosity with different water-to-powder ratios. J Endod 2003;29:814–7.
13. Thomson TS, Berry JE, Somerman MJ, Kirkwood KL. Cementoblasts maintain expression of osteocalcin in the presence of mineral trioxide aggregate. J Endod 2003;29:407–12.
14. Abdullah D, Pitt Ford TR, Papaioannou S, Nicholson J, McDonald F. An evaluation of accelerated Portland cement as a restorative material. Biomaterials 2002;23:4001–10.
15. Holland R, Souza V, Nery MJ, Otoboni JA, Filho Bernabe PFE, Dezan E Jr. Reaction of rat connective tissue to implanted dentin tubes field with mineral trioxide aggregate or calcium hydroxide. J Endod 1999;25:161–6.
16. Mitchell PJC, Pitt Ford TR, Torabinejad M, McDonald F. Osteblast biocompatibility of mineral trioxide aggregate. Biomaterials 1999;20:167–73.
17. Koh ET, McDonald F, Pitt Ford TR, Torabinejad M. Cellular response to mineral trioxide aggregate. J Endod 1998;24:543–7.
18. Koh ET, Torabinejad M, Pitt Ford TR, Brady K, McDonald F. Mineral trioxide aggregate stimulates a biological response in human osteoblasts. J Biomed Mat Res 1997;37:432–9.
19. Torabinejad M, Hong CU, Lee SF, Monsef M, Pitt Ford TR. Investigation of mineral trioxide aggregate for root-end-filling in dogs. J Endod 1995;21:603–8.
20. Wucherpfennig AL, Green DB. Mineral trioxide vs Portland cement: two biocompatible filling materials [abstract]. J Endod 1999;25:308
21. Estrela C, Bahmann LL, Estrela CRA, Silva RS, Pécora JD. Antimicrobial and chemical study of MTA, Portland cement, calcium hydroxide paste, Sealapex and Dycal. Braz Dent J 2000;11:19–27.
22. Menezes R, Bramante CM, Letra A, Carvalho VGG, Garcia RB. Histologic evaluation of pulpotomies in dog using two types of mineral trioxide aggregate and regular and White Portland cements as wound dressings. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;98:376–9.
23. Auletta A, Ashby J. Workshop on the relationship between short-term information and carcinogenicity; Williamsburg, VA, January 20–23, 1987. Environ Mol Mutagen 1988;11:135–45.
24. Tice RR, Agurell E, Anderson D, et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 2000;35:206–21.
25. Alves A, De Miranda Cabral Gontijo AM, Salvadori DM, Rocha NS. Acute bacterial cystitis does not cause deoxyribonucleic acid damage detectable by the alkaline comet assay in urothelial cells of dogs. Vet Pathol 2004;41:299–301.
26. Ladeira MS, Rodrigues MA, Salvadori DM, Queiroz DM, Freire-Maia DV. DNA damage in patients infected by Helicobacter pylori. Cancer Epidemiol Biomarkers Prev 2004;13:631–7.
27. Ribeiro DA, Bazo AP, Franchi CAS, Marques MEA, Salvadori DM. Chlorhexidine induces DNA damage in rat peripheral leukocytes and oral mucosal cells. J Periodont Res 2004;39:358–61.
28. Ribeiro DA, Marques MEA, Salvadori DMF. Lack of genotoxicity of formocresol, paramonochlorophenol and calcium hydroxide on mammalian cells by comet assay. J Endod 2004;30:593–6.
29. Ribeiro DA, Scolastici C, Marques MEA, Salvadori DMF. Fluoride does not induce DNA breakage in Chinese hamster ovary cells in vitro. Braz Oral Res 2004;18:192–6.
30. Rothfuss A, Merck O, Radermacher P, Speit G. Evaluation of mutagenic effects of hyperbaric oxygen (HBO) in vitro II. Induction of oxidative DNA damage and mutations in the mouse lymphoma assay. Mutat Res 2000;471:87–94.
31. Mckelvey-Martin VJ, Green MHL, Schmezer P, Pool-Zobel BL, De Méo MP, Collins A. The single cell gel electrophoresis assay (comet assay): a European review. Mutat Res 1993;288:47–63.
32. Hartmann A, Agurell E, Beevers C, et al. Recommendations for conducting the in vivo alkaline comet assay. Mutagenesis 2003;18:45–51.
33. Geurtsen W. Substances released from dental resin composites and glass ionomer cements. Eur J Oral Sci 1998;106:687–95.
34. Asrari M, Lobner D. In vitro neurotoxic evaluation of root-end-filling materials. J Endod 2003;29:743–6.
35. Keiser K, Johnson CC, Tipton DA. Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. J Endod 2000;26:288–91.
36. Osório RM, Hefti A, Vertucci FJ, Shawley AL. Cytotoxicity of endodontic materials. J Endod 1998;24:91–6.
37. Glasser FP. Reactions occurring during cement making. In: Barnes P et al. Structure and Performance of Cements. New York: Applied Sciences Publishers, 1983:104–5.
38. Ribeiro DA, Pereira PC, Machado JM, Silva SB, Pessoa AW, Salvadori DM. Does toxoplasmosis cause DNA damage? An evaluation in isogenic mice under normal diet or dietary restriction. Mutat Res 2004;559:169–76.

