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In vitro biocompatibility tests of two commercial types of mineral trioxide aggregate

Testes de biocompatibilidade in vitro *de duas formas comerciais do agregado de trióxido mineral*

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ABSTRACT: Recently, regular and white mineral trioxide aggregate (MTA) are being used in Dentistry as retrofilling materials. Genotoxicity and cytotoxicity tests form an important part of cancer research and risk assessment of potential carcinogens. Thus, the goal of this study was to examine the genotoxicity and cytotoxicity of regular and white MTA *in vitro* by the single cell gel (comet) assay and trypan blue exclusion test, respectively. Mouse lymphoma cells were exposed to two presentation forms of MTA at final concentrations ranging from 1 to 1,000 μg/mL for 3 h at 37°C. The results showed that both compounds tested did not produce genotoxic effects at all concentrations evaluated. Likewise, no statistically significant differences ($p > 0.05$) were observed in cytotoxicity. Taken together, our results suggest that regular and white MTA are not genotoxins and are not able to interfere in cellular viability as assessed by single cell gel (comet) assay and trypan blue assay, respectively.

DESCRIPTORS: Mineral trioxide aggregate; Genotoxicity tests; Comet assay; Trypan blue; Mouse lymphoma cells.

RESUMO: Recentemente, o agregado de trióxido mineral (MTA) regular e branco estão sendo utilizados na Odontologia como materiais para obturação retrógrada de canais radiculares. Testes de genotoxicidade e citotoxicidade formam uma importante parte da pesquisa do câncer e da avaliação de risco de carcinógenos potenciais. Assim, o objetivo deste estudo foi examinar a genotoxicidade e citotoxicidade do MTA branco e regular *in vitro* pelo teste do cometa e teste de exclusão pelo azul de tripan, respectivamente. Células do linfoma murino foram expostas às duas formas de apresentação do MTA nas concentrações finais de 1 a 1.000 μg/mL por 3 horas a 37°C. Os resultados mostraram que ambos os compostos testados não produziram efeito genotóxico em todas as concentrações testadas. Da mesma forma, nenhuma diferença estatisticamente significativa ($p > 0,05$) foi observada na citotoxicidade. Em suma, nossos resultados sugerem que o MTA regular e branco não são genotoxinas e não são capazes de interferir na viabilidade celular conforme avaliado pelo teste do cometa e ensaio do azul de tripan, respectivamente.

DESCRITORES: Mineral trióxido agregado; Testes de genotoxicidade; Teste de cometa; Azul tripano; Células do linfoma murino.

INTRODUCTION

Biocompatibility is the ability of a material to be used for a specific application without having toxic or injurious effects on biological function. In this context, such material should be easy to manipulate, radiopaque, dimensionally stable, non-absorbable, and nontoxic[4]. In the 1990s, a new material, mineral trioxide aggregate (MTA) (which is grey in colour) was developed as a retrofilling material. Herein, a number of biocompatibility studies have been conducted either *in vitro* or *in vivo*, and the results showed that MTA presents good sealing ability and tissue healing[7,9,10,12,13,24-27]. Recently, a new tooth-coloured form of MTA has been developed for use in endodontic practice in order to fulfill esthetic recommendations[16]. However, further biocompatibility data are needed to evaluate the risk of using these compounds[2].

Taking into account the biocompatibility tests available in general, genotoxicity assays are of special concern since genotoxicity has gained widespread acceptance as an important and useful indicator of carcinogenicity[1]. A variety of assays

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can assess genotoxicity, including those that assess metaphase chromosomal aberrations, micronuclei, sister chromatid exchanges and host cell reactivation. However, these methods are typically laborious and time-consuming or require highly trained technicians to accurately read and interpret slides. In the past decade, the single cell gel (comet) assay in alkaline version was developed. It is a rapid, simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells[23]. 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[14]. Previous studies conducted by our group have proved that single cell gel (comet) assay is a suitable experimental model to test genotoxicity of compounds used in dental practice[17,18,20,21].

Therefore, the purpose of this study was to investigate whether two commercial forms of MTA can induce DNA breakage in mouse lymphoma cells by the single cell gel (comet) assay. To monitor cytotoxic effects, the trypan blue exclusion test was used. These results will contribute to a better understanding of the mechanism of action of dental materials upon the cellular system.

MATERIALS AND METHODS

Cell culture

L5178Y mouse lymphoma cells were cultivated in suspension in RPMI 1640 glutamax medium (Life Sciences, New York, USA) supplemented with 10% heat-inactivated horse serum and penicillin/streptomycin (Life Technologies, New York, USA) at 37°C with 5% CO_2 according to Rothfuss *et al.*[22] (2000). Mouse lymphoma cells were first defrosted and subsequently sub-cultivated three times before performing the experiment. Cell suspension was counted using a Neubauer® chamber (Kerka, Germany) and seeded in 96-well microtitre plated (Corning, NY, USA) at a density of 1×10^4 cells per well (at a concentration of 1×10^6/mL). All the procedures in this study were in accordance with the ethical conducts described by the Committee of the School of Medicine of Botucatu, São Paulo State University.

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 1,000 µg/mL. The negative control group was treated with vehicle control (PBS) and the positive control group was treated with methyl methanesulfonate (MMS at 10 µg/mL, Sigma Aldrich, St. Louis, USA). After incubation for 3 h at 37°C, the cells were centrifuged at 1,000 rpm (180 g) during 5 min and washed twice with fresh medium (Invitrogen Corporation, New York, USA) and resuspended with fresh medium. Each individual treatment was repeated three times consecutively to ensure reproducibility.

Cytotoxicity assay

Cytotoxicity assay was performed using Trypan blue (Sigma Aldrich, St. Louis, USA) staining after the treatment[11]. In brief, a freshly prepared solution of 10 µl Trypan blue (0.05%) in distilled water was mixed to 10 µl of each cellular suspension during 5 min, spread onto a microscope slide (Bioglass, Taubaté, Brazil) and covered with a coverslip. Non-viable 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 proposed by Tice *et al.*[23] (2000). In brief, a volume of 10 µl of treated or control cells (approximately 1×10^4 cells) were added to 120 µl of 0.5% low-melting point agarose (Invitrogen Corporation, New York, USA) at 37°C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in the refrigerator, the coverslip was removed and slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA – Merck, St. Louis, USA; 10 mM Tris-HCl buffer pH = 10 – Sigma Aldrich, St. Louis, USA; 1% sodium sarcosinate – Sigma Aldrich, St. Louis, USA; with 1% Triton X-100 – Sigma Aldrich, St. Louis, USA; and 10% dimethyl sulfoxide (DMSO) – Merck, St. Louis, USA) for about 1 hour. Prior to electrophoresis, the slides were left in alkaline buffer (0.3 mM NaOH, Merck, St. Louis, USA; and 1 mM EDTA, Merck, St. Louis, USA; pH > 13) for 20 min and electrophoresed for another 20 min, at 25 V (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 (Merck, Darmstadt, Germany) 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[6] were examined blindly at 400 X magnification using a fluorescence microscope (Olympus, Orangeburg, USA) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Sufolk, 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

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

RESULTS

The cytotoxicity of MTA (grey and white) was measured in mouse lymphoma 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 1-1,000 μg/mL on cell viability assessed by trypan blue assay are shown in Table 1. No significant statistically differences ($p > 0.05$) of viable cells were found for both endodontic materials tested and in all concentrations tested.

The single cell gel (comet) assay was used to measure DNA damage in mouse lymphoma cells *in vitro*. DNA strand breaks were represented by the mean tail moment for 50 comets/sample. As seen in Table 2, both materials did not induce DNA strand breaks at any concentration tested.

TABLE 1 - Effects of serial concentrations of regular and white MTA assessed by trypan blue exclusion test. Results are expressed as mean percentage of the control (mean ± standard deviation).

Concentration (μg/mL)	Regular MTA	White MTA
1,000	88.00 ± 3.00	87.67 ± 3.05
100	89.67 ± 3.21	92.34 ± 3.78
10	90.34 ± 3.51	89.00 ± 2.64
1	92.00 ± 3.00	93.66 ± 3.21
Negative control[1]	94.67 ± 3.05	94.67 ± 3.05

[1]Phosphate buffer solution (pH 7.4).

TABLE 2 - Mean ± Standard deviation of DNA damage (tail moment) in mouse lymphoma cells exposed to regular and white MTA.

Concentration (μg/mL)	Regular MTA	White MTA
1,000	0.71 ± 0.36	0.58 ±0.33
100	0.75 ± 0.25	0.83 ±0.19
10	0.41 ± 0.17	0.77 ±0.24
1	0.74 ± 0.18	0.61 ±0.17
Negative control[1]	0.59 ± 0.16	0.59 ±0.16
Positive control[2]	4.83 ± 1.20*	4.83 ±1.20*

[1]Phosphate buffer solution (pH 7.4); [2]Methyl methanesulfonate at 10 μg/mL; *$p < 0.05$ when compared to negative control.

DISCUSSION

In this study, a cell culture technique was employed in order to evaluate the biocompatibility for two forms of MTA. *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[5]. The results obtained from *in vitro* assays might be indicative of the effects observed *in vivo*. L5178Y, a continuous cell line, was used as the target cell in this experiment. Cell lines are easier to prepare and culture than primary cells (lymphocytes from peripheral blood). Primary cells are used in clinically simulated situations but are rather different between individuals. Our own most recent findings have shown that the two cell types do not differ much in sensitivity[18].

Introduction of chemicals in the working environment requires the assessment of their harmful effects. The trypan blue exclusion test can be used

to indicate cytotoxicity; dead cells take up the blue stain of trypan blue, whereas live cells have yellow nuclei. Cytotoxicity data obtained in our laboratory on mouse lymphoma cultures demonstrated that either regular or white MTA were not able to interfere in cellular death at any concentration assessed. This is consistent with published data reporting that MTA has been found to be non-toxic *in vitro*[8,15,25].

The single cell gel (comet) assay is a sensitive method for the detection of DNA damage and repair induced by genotoxic compounds in individual level. The alkaline version, used in this study, is able to detect a variety of DNA lesions and incomplete repair sites[3,23]. Therefore, and taking into account the lack of data currently available, the assessment of the potential genotoxicity of MTA is justified. The results of this study indicated that the alkaline single cell gel (comet) assay, in the experimental conditions used, failed to detect the presence of DNA damage after treatment using both forms of MTA assessed at any concentration tested. These findings confirmed and extended the data already published, showing that MTA presents good biocompatibility[7,12].

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 based on the assumption that these cells represent dead cells, resulting from putative cytotoxic effects of root-end filling materials rather than primary DNA-damage following direct interaction between DNA and a genotoxic agent[19].

CONCLUSION

In summary, our results suggest that both regular and white MTA are not genotoxins and are not able to interfere in cellular death. Furthermore, the results presented here might be an additional argument to support the use of MTA in endodontic practice.

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