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ORIGINAL ARTICLE

Evaluation of the Genotoxicity of Endodontic Materials for Deciduous Teeth Using the Comet Assay

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ABSTRACT

Objective: To evaluate genotoxicity of zinc oxide, P. A. calcium hydroxide, mineral trioxide aggregate and an iodoform paste using comet assay on human lymphocytes. **Material and Methods:** Two positive controls were used: methyl-methanesulfonate for the P.A. calcium hydroxide and mineral trioxide aggregate; and doxorubicin for the iodoform paste and zinc oxide. There were also two negative controls: distilled water for the P.A. calcium hydroxide and mineral trioxide aggregate; and DMSO for the iodoform paste and zinc oxide. Comets were identified using fluorescence microscopy and 100 of them were counted on each of the three slides analyzed per drug test. A damage index was established, taking into consideration the score pattern that had previously been determined from the size and intensity of the comet tail. Analysis of variance, followed by Tukey's test, was used to compare the means of the DNA damage indices. **Results:** The DNA damage index observed for mineral trioxide aggregate (7.08 to 8.58) and P.A. calcium hydroxide (6.50 to 8.33), which were similar to negative control index. On the other hand, damage index for zinc oxide (104.7 to 218.50) and iodoform paste (115.7 to 210.7) were similar to positive control index. **Conclusion:** Iodoform paste and zinc oxide showed genotoxicity at all concentrations used.

Keywords: Endodontics; Tooth, Deciduous; Mutagenicity Tests; Cells, Cultured.

Introduction

Dental caries and dentoalveolar trauma can cause irreversible alterations to dental pulp, culminating in necrosis, which can lead to teeth loss. In view of these changes, pulp therapy is the last resource for deciduous tooth preservation under functional conditions until physiological exfoliation. It should be emphasized that, for successful pulp therapy, appropriate root canal treatment is necessary, using chemical-mechanical preparation along with hermetic filling [1,2].

Among the filling materials for deciduous teeth are used zinc oxide and eugenol paste, iodoform pastes, calcium hydroxide-based paste and mineral trioxide aggregate paste. Although pulp therapy of primary teeth is used for many decades, there is no consensus on the filling paste that could be considered as an ideal material. To deciduous pulp therapy, the paste must be resorbable, provide radiopacity, not interfere with the successor tooth germ's development, and be biocompatible with periapical tissue [1,3-5]. Additionally, it is expected that an ideal filling material does not induce genotoxic and/or cytotoxic effects, but also there's also no consensus on such effects in the studies [6-17].

Genotoxicity of mineral trioxide aggregate (MTA) and calcium hydroxide P.A. (pro-analysis) has not been reported, with no greater occurrence of DNA damage [6,9,12,18,19]. Concerning the potential of products containing zinc oxide or iodoform pastes, there is a scarcity of studies, especially those with the comet methodology. Pires et al. [19] showed that comet assay revealed that iodoform paste did not damage DNA either Guedes Pinto paste, but Santos et al. [12] found genotoxic potential, translated as a higher frequency of micronuclei.

Evaluation of the potential of a product with regard to inducing DNA damage is of great importance, given the relationship between mutagenicity and carcinogenesis [16,17]. Among the tests available for such evaluations include the comet assay [20,21], which has also been used for biomonitoring of individuals or populations when exposed to mutagens [22]. This assay detects single and double stand breaks in DNA molecules caused by alkylating, intercalating and oxidant agents and it can be performed on animal and vegetable cells, both *in vitro* and *in vivo*, and it is a methodology that has most often been employed to assessment of the genotoxicity filling pastes [6,9,18,23-26].

The objective of the present study was to evaluate human lymphocytes, using the comet assay, regarding the *in vitro* genotoxicity of pro-analysis calcium hydroxide (PACH), mineral trioxide aggregate (MTA), zinc oxide and an iodoform paste as recommended by Guedes-Pinto, Paiva and Bozzola [26].

Material and Methods

Ethical Approval

This research was approved by the Ethics Committee of the Federal University of Ceará, Brazil, under COMEPE-UFC protocol number 281/09.

Chemical Substances

Four filling paste preparations were used: mineral trioxide aggregate (MTA; Angelus Soluções Odontológicas, Londrina, Brazil); calcium hydroxide P.A. (PACH; Biodinâmica Produtos e Serviços para Laboratórios Ltda, Ibiporá, Brazil) and zinc oxide (ZO; Dentsply, Petrópolis, Brazil). Iodoform paste (IP), in accordance with dental practice, was prepared in the laboratory by mixing equal parts of iodoform (Biodinâmica Produtos e Serviços para Laboratórios Ltda, Ibiporá, PR, Brazil), camphorated paramonochlorophenol (Biodinâmica Produtos e Serviços para Laboratórios Ltda, Ibiporá, PR, Brazil) and an

ointment composed of 0.15% rifamycin and 0.5% prednisolone. Methyl methanesulfonate (MMS) or doxorubicin were used as a positive control and dimethyl sulfoxide (DMSO) or distilled water as a negative control.

Cell Culture

Heparinized blood (from healthy, non-smoker donors who had not taken any drug at least 15 days prior to sampling) was collected and peripheral blood mononuclear cells (PBMC) were isolated by a standard method of density-gradient centrifugation over Histopaque-1077. PBMC were washed and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin at 37°C with 5% CO_2. Phytohemagglutinin (3%) was added at the beginning of culture. After 24 h of culture, cells were treated with the test compounds.

Test Sample Dilutions

Two test samples (ZO and IP) in the form of powder and paste respectively were diluted in DMSO and two (PACH and MTA) in the form of powder in distilled water, at the following concentrations: 1:500, 1:750, 1:1000 and 1:2000. The alkylating compound methyl methanesulfonate was the reference for IP and ZO and was used at a concentration of 0.4 µM diluted in distilled water (v/v). Doxorubicin was used as a positive control for HPCH and MTA, at a concentration of 0.6µM diluted in DMSO at 0.1% (v/v).

Comet Assay

The comet assay, used to detect DNA strand breaks, was conducted under alkaline conditions as described by Singh et al. [20] with minor modifications [27] and following the recommendations of the International Workshop on Genotoxicity Test Procedures [22]. At the end of the treatment, PBMC were washed with ice-cold PBS, trypsinized with 100 µL trypsin (0.15%) and resuspended in a complete RPMI medium. Then, 20 µL of cell suspension (~106 cells/mL) were dissolved in 0.75% low melting point agarose and immediately spread onto a glass microscope slide precoated with a layer of 1% normal melting point agarose. The slides were incubated in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH = 10.0) at 4°C for a minimum of 1 h. After then, they were placed on a horizontal electrophoresis unit filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH >13.0) for 20 min at 4°C to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25V and 300mA (0.86V/cm). Each slide was stained with 50 µL of ethidium bromide (20 µg/mL) and covered with a coverslip. The cells' analysis was performed by a visual scoring system using an epifluorescence microscope (Olympus, Tokyo, Japan) with an excitation filter of 510–560 nm and a barrier filter of 590 nm at 400x magnification [28].

Three hundred randomly selected cells (100 cells from each of the three replicate slides) were analyzed for each concentration of test substance. Cells were scored visually according to tail length into five classes: (1) class 0: undamaged cells having no tail; (2) class 1: cells having a tail shorter than the diameter of the head (nucleus); (3) class 2: cells having a tail length 1–2 times the diameter of the head; (4) class 3: cells having a tail longer than 2 times the diameter of the head; (5) class 4: comets having no heads. For the data analysis, the slides were coded; decoding was done after genotoxicity determination by one (single) experienced blinded investigator standardized with the team of the Toxicological Genetics Laboratory of the Federal University of Ceará.

Statistical Analysis

A value (damage index, DI) was assigned to each comet according to its class, using the formula: DI = (0 x n0) + (1 x n1) + (2 x n2) + (3 x n3) + (4 x n4), where n = number of cells in each class analyzed. Damage index thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). The damage frequency (DF) represents the percentage of cells that suffered DNA damage. For statistical analysis on the experiments, ANOVA and Tukey test were used in the Prisma software, version 6.0 (GraphPad Prism Software). Results were considered to be statistically significant when $p < 0.05$.

Results

Table 1 presents the average rate of DNA damage (DR) and their mean standard error (SEM) found for the test samples of negative and positive controls: distilled water and methyl methanesulfonate (MMS), respectively; calcium hydroxide P.A. (PACH) and mineral trioxide aggregate (MTA). Statistical analysis revealed no significant differences between the mean values obtained in the different concentrations of the test samples and negative controls, which were significantly lower than that calculated for the positive control.

Table 1. Data on average rate of DNA damage (DR), and their mean standard error (SEM).

Samples	Treatment	DR ± SEM[a]
Distilled Water		5.66 ± 0.73[a]
Methyl Methane Sulfonate	0.4 µM	178.75 ± 5.90[b]
P. A Calcium Hydroxide	1:500	6.83 ± 1.00 [a]
	1:750	8.33 ± 0.84 [a]
	1:1000	7.91 ± 0.99 [a]
	1:2000	6.50 ± 1.09 [a]
Mineral Trioxide Aggregate	1:500	7.66 ± 0.87 [a]
	1:750	8.58 ± 0.89 [a]
	1:1000	7.58 ± 0.98 [a]
	1:2000	7.08 ± 0.94 [a]

Different letters in the same column, the treatments differ significantly by Tukey test ($p<0.05$).

Table 2 presents the average rates of DNA damage (ID) and standard error of the mean (SEM) calculated for the test samples of negative and positive controls: dimethylsulfoxide (DMSO) and doxorubicin (DOX), respectively; zinc oxide (ZO) and Iodoform Paste, respectively. Statistical analysis revealed that the mean levels of DNA damage obtained from the treatment of test samples (ZO and IP) were significantly higher than the negative control and positive control did not differ from average.

Tabela 2. Data on average rate of DNA damage (DR), and their mean standard error (SEM).

Samples	Treatment	DR ± SEM[a]
DMSO	0.1% (v/v)	14.00 ± 0.57[a]
Doxorubicin	0.6 µM	145.7 ± 32.33[b]
Zinc Oxid	1:500	218.5 ± 7.57[b]
	1:750	204.0 ± 13.20[b]
	1:1000	143.5 ± 12.14[b]
	1:2000	104.7 ± 9.33[b]
Iodoform Paste	1:500	210.7 ± 30.57[b]
	1:750	153.5 ± 26.12[b]
	1:1000	123.3 ± 9.68[b]
	1:2000	115.7 ± 12.93[b]

Different letters in the same column, the treatments differ significantly by Tukey test ($p<0.05$).

Discussion

The present study evaluated the genotoxicity of filling pastes for deciduous tooth pulpectomy that is a matter of great relevance for pediatric dentistry when analyzing the relation of the product regarding the mutagenic potential. The paste options currently available are iodoform-based, calcium hydroxide-based, zinc oxide-based (with or without eugenol) and mineral trioxide aggregate-based [29,30]. These products were investigated in the present study due to their use in dental practice and the scarcity of studies in the literature addressing these substances' genotoxic potential. This study used the comet assay to access the genotoxic potential of filling pastes. This is a sensitive and quick method that is able to detect DNA injuries in individual cells through direct measurement [31,32]. This method has been used with some frequency to evaluate the genotoxicity of cements and root canal filling pastes currently used in pulp therapy. This study revealed that zinc oxide, tested alone without eugenol and iodoform paste was genotoxic at all tested concentrations. In the literature, Pires et al. [19] inferred the same to the iodoform paste; however, Santos et al. [12] found different result and verified that these pastes (IP and OZ) both showed genotoxicity, translated like micronuclei, and cytotoxicity expressed by relation polychromatic erythrocyte and normochromic erythrocytes (PCE/NCE).

Regarding zinc oxide, some studies also performed comet assay on human lymphocytes, but the dental filling products tested were zinc oxide-based cement with and without eugenol and not the pure paste tested used in our study. Moreover, the treatments were different, such that these authors used lower concentrations. Under these conditions, they concluded that the substances evaluated presented "acceptable biocompatibility in terms of genotoxicity" [8,25,33].

Conflicting results regarding zinc oxide's genotoxicity have been reported in studies in which the endpoints analyzed were chromosome damage [25,33]. While Hikiba et al. [33] observed a greater occurrence of chromosome aberrations in embryonic hamster cells in the cultures treated, Brzovic et al. [25] did not report any greater occurrence of these aberrations in cultures of human lymphocytes treated with zinc oxide-based cement and eugenol. Evaluating chromosome damage translated by micronuclei, Camargo et al. [8] did not report any greater occurrence of these structures in fibroblasts from Chinese hamsters that were treated with cement-based on this substance.

No genotoxic effects from iodoform were reported by Hikiba et al. [33]. Such effects would have been translated through greater occurrence of chromosome aberrations in embryonic Chinese hamster cells. The results obtained by Hagiwara et al. [34] and Ribeiro et al. [18,24], from evaluating the potential of paramonochlorophenol for inducing chromosome aberrations and DNA damage, respectively, did not indicate any potential for genotoxic effects. The induction of DNA damage by iodoform paste revealed in the present study provides support for conducting new investigations and constitutes an initial alert regarding whether it should be indicated.

The results from the present study are in agreement with those of da Silva et al. [6] and Braz et al. [24], who evaluated the genotoxicity of MTA through using the comet assay on human lymphocytes and also did not observe any greater occurrence of DNA damage. This paste has been investigated by several other authors, using the comet assay on other cell types, and except for the results obtained by Naghavi et al. [11], no genotoxic effects were identified in any of them [6,9,16,24]. Similar results were showed by Santos et al. [12], using Micronuclei Test.

In the same way as in the present study, no genotoxic effects from calcium hydroxide were identified through the comet assay by Brzovic et al. [25], similarly to results founded by Santos et al. [12] using a different methodology. In the studies by Ribeiro et al. [35,36], the potential of this substance for inducing

DNA damage was evaluated using the comet assay on lymph cells from mice (L5178Y) and ovary cells from Chinese hamsters (K-1), respectively. In these cell types, too, no greater occurrence of DNA damage was described.

Thus, the results obtained through using the comet assay have revealed that calcium hydroxide does not induce DNA damage. Regarding the potential for calcium hydroxide to induce chromosome damage, Camargo et al. [8] reported a greater occurrence of micronuclei in fibroblasts from Chinese hamsters (lineage V79) that were treated with an endodontic cement based on this substance but which also contained glycyrrhizic acid, methenamine and bisphenol-A. Thus, the genotoxic effects observed could not be attributed in isolation to one of the components of this cement.

From the foregoing, it can be supposed that different biological systems evaluated in the same test and different endpoints evaluated through different methodologies are important factors that lead to conflicting results in evaluating a given product's genotoxicity.

It also has to be taken into consideration that discrepant results may be due to different protocols used for performing the same test. In this context, a few comments are needed in relation to some aspects of the methodology used in the present study. Firstly, the decision to use a single donor to obtain lymphocytes was based on the study by Vijayalaxmi et al. [32] and also on the protocol used in the National Experimental Oncology Laboratory of the Department of Physiology and Pharmacology, Federal University of Ceará, which is the place where the comet assay was developed.

In relation to visual analysis, Wong et al. [31] and Dhawan et al. [37] considered that both types of analysis were valid. According to Collins [38], faithful assessment of the different degrees of DNA damage in accordance with the appearance of the comet does not necessarily require image analysis software, given that the human eye can easily be trained to recognize the characteristics involved. This kind of evaluation was chosen [39,40] and the comet analysis was performed by an experienced blinded investigator in triplicate with coded slides as recommended by Olive and Banáth [21], Hartmann et al. [41] and Wong et al. [31].

Conclusion

Zinc oxide and iodine paste showed genotoxicity translated with DNA damage according to the comet assay. Calcium hydroxide and mineral trioxide aggregate showed no DNA damage.

Authors' Contributions

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EMMC	https://orcid.org/0000-0003-1047-4931	Methodology, Validation, Formal Analysis, Investigation and Writing - Review and Editing.

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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