Braz Dent J (2006) 17(3): 228-232 ISSN 0103-6440

In Vitro Cytotoxic and Non-Genotoxic Effects of Gutta-Percha Solvents on Mouse Lymphoma Cells by Single Cell Gel (Comet) Assay

Daniel Araki RIBEIRO
Mariângela Esther Alencar MARQUES
Daisy Maria Fávero SALVADORI

Center for Genotoxin and Carcinogen Evaluation (TOXICAN), Department of Pathology, School of Medicine of Botucatu, São Paulo State University, Botucatu, SP, Brazil

Chloroform and eucalyptol are widely used in clinical dentistry as gutta-percha solvents. However, these compounds may represent a hazard to human health, especially by causing injury to genetic apparatus and/or inducing cellular death. In this study, the genotoxic and cytotoxic potentials associated with exposure to chloroform and eucalyptol were assessed on mouse lymphoma cells *in vitro* by the single cell gel (comet) assay and trypan blue exclusion test, respectively. Both gutta-percha solvents proved to be cytotoxic at the same levels in concentrations of 2.5, 5 and 10 µL/mL ($p<0.05$). On the other hand, neither of the solvents induced DNA breakage. Taken together, these results suggest that although both tested compounds (chloroform and eucalyptol) are strong cytotoxicants, it seems that they are not likely to increase the level of DNA damage on mammalian cells.

Key Words: chloroform, eucalyptol, mouse lymphoma cells, genotoxicity, cytotoxicity.

INTRODUCTION

Gutta-percha has been used in Endodontics for over 100 years and is currently the most frequently used core material for permanent obturation of root canals (1). However, when root canal therapy fails, the tooth should undergo endodontic re-treatment, which basically consists of the removal of gutta-percha cones from inside the root canals. There are several methods for gutta-percha removal: thermal, mechanical and chemical solvents. Chloroform is one of the most widely used inorganic solvents for softening or dissolving gutta-percha. However, side effects from exposure to chloroform have been reported (2). Furthermore, studies have addressed that chloroform is possibly carcinogenic to humans (3,4). These concerns about chloroform have renewed the interest in finding alternative solvents (5-7). Eucalyptol, a widely used substance for flavoring and fragrance, has been used herein as a solvent without apparent harmful effects (8).

Understanding how cancer develops creates opportunities for prevention or early detection. An important part of this effort is to identify the agents and exposures that potentially cause cancer. Genotoxicity assays can be defined as *in vitro* and *in vivo* tests designed to detect compounds that might induce genetic damage such as DNA strand breaks, gene mutation, chromosomal breakage and altered DNA repair capacity. In the last decades, genotoxicity assays have gained widespread acceptance as an important and useful indicator of carcinogenicity (9).

While it has been clearly demonstrated that chloroform is a putative carcinogen for humans, the number of studies on the genotoxic effect of chloroform and other gutta-percha solvents, i.e., eucalyptol, is still limited. The comet assay is a relatively new, rapid, simple technique for evaluating DNA damage in mammalian cells (10). This technique includes embedding cells in agarose gel onto microscope slides, incubating them with the test compound and then lysing the cells

Correspondence: Dr. Daniel Araki Ribeiro, Núcleo de Avaliação Toxicogenética e Cancerígena (TOXICAN), Departamento de Patologia, Faculdade de Medicina de Botucatu, UNESP, Distrito de Rubião Jr. S/N, 18618-000 Botucatu, SP, Brasil. Tel: +55-14-3882-8255. Fax:+55-14-3815-2348. e-mail: araki@fmb.unesp.br

with detergent and high salts. During electrophoresis under alkaline conditions, cells with damaged DNA display increased rates of DNA migration to the anode and this picture resembles a 'comet' with brightly fluorescent head and a tail region (10). Therefore, the purpose of this study was to evaluate *in vitro* the genotoxic effects of chloroform and eucalyptol in mouse lymphoma cells by the single cell gel (comet) assay. To monitor cytotoxic effects, Trypan blue exclusion test was performed.

MATERIAL AND METHODS

All procedures in this study were approved by and performed in compliance with the Ethics Committee of the Faculty of Medicine of Botucatu, UNESP, Brazil.

Cell Culture. L5178Y mouse lymphoma cells were cultivated in suspension in RPMI 1640 glutamax medium (Amersham Life Sciences Inc., Arlington Heights, IL, USA) supplemented with 10% heat-inactivated horse serum and penicillin/streptomycin (Life Technologies, Grand Island, NY, USA) at 37°C with 5% CO_2 according to Rothfuss et al. (11). 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 microtiter plates (Corning Inc., Corning, NY, USA) at a density of 1×10^4 cells *per* well (at a concentration of 1×10^6/mL).

Cell Treatment. The tested materials were: chloroform (Merck, Darmstadt, Germany) and eucalyptol (Inodon, Laboratório Ltda, Porto Alegre, RS, Brazil). Both materials were prepared in final concentrations ranging from 1.25 to 10 µL/mL. The negative control group was treated with a vehicle control (Phosphate-Buffered Saline - PBS) and the positive control group was treated with methyl methanesulfonate (MMS) at 10 µL/mL (Sigma Aldrich, St. Louis, MO, USA). After incubating at 37°C for 3 h, the cells were centrifuged at 1000 rpm (180 G) during 5 min, washed twice with fresh medium and re-suspended with fresh medium. Each individual treatment was repeated 3 times consecutively to ensure reproducibility.

Cytotoxicity Assay. Cell viability test for mouse lymphoma cells was performed using Trypan blue staining before treatment (12). Namely, a freshly prepared solution of 10 µL Trypan blue (0.05%) in distilled water was mixed to 10 µL of each cell suspension during 5 min, spread onto a microscope slide and covered with a coverslip. Non-viable cells were 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. (10). Briefly, 10 µL of treated or control cells (~1×10^4 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 refrigerator, the coverslip was removed and the slides were immersed in a lysing solution consisting of 2.5 M sodium chloride, 100 mM ethylenediaminetetraacetic (EDTA) (Merck), 10 mM Tris-HCl buffer at pH 10 (Sigma Aldrich), 1% sodium sarcosinate (Sigma Aldrich) with 1% Triton X-100 (Sigma Aldrich) and 10% dymethylsulfoxide (DMSO) (Merck) for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer containing 0.3 mM NaOH (Merck) and 1 mM EDTA (Merck) (pH>13) for 20 min and electrohoresed 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 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 (13) were examined blindly at X400 magnification using a fluorescence microscope (Olympus, Orangeburg, NY, USA) connected to a black and white camera. Comet Assay II automated analytical software (Perceptive Instruments, Sufolk, Haverhill, UK) was used. The computed image analysis system acquired the images, computed the integrated intensity profiles for each cell, estimated the comet cell components and then evaluated 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 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 each sample was taken as an index of DNA damage in this sample.

Statistical Methods. Parameters from the cytotoxicity and the single cell gel (comet) assay for treated

cells versus control cells were assessed by 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 gutta-percha solvents was measured in mouse lymphoma cells by means of trypan blue assay in range-finding experiment prior to determination of chemically induced genotoxicity. The percentages of viable cells exposed to chloroform or eucalyptol are shown in Figure 1. At 2.5, 5 and 10 µL/mL concentrations, the percentage of cell viability for both gutta-percha solvents were statistically significant ($p<0.05$) when compared to the negative control. However, at a concentration of 1.25 µL/mL, no statistically significant difference ($p>0.05$) was detected and more viable cells were found in chloroform than in eucalyptol.

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 *per* sample. Neither of the solvents induced DNA strand breaks (Table 1).

DISCUSSION

In this study, a cell culture technique was employed to evaluate the biocompatibility of gutta-percha solvents. *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 (14). The results obtained from *in vitro* assays might be indicative of the *in vivo* effects. L5178Y 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 these two cell types do no differ much in their comparative sensitivity (15).

Introduction of chemicals in the working environment requires the assessment of their harmful effects. Minimal progress in biocompatibility *in vitro* tests concerning cytotoxicity and/or genotoxicity of gutta-percha solvents has been achieved over the past decade, and few investigations in the field of dental research have taken full advantage of the several endpoint assays that have been developed in the field of general research to investigate different compounds.

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. In this study, cytotoxicity data obtained from mouse lymphoma cultures demonstrated that chloroform was able to produce cellular death in a dose-related fashion, the strongest effect being observed at higher concentrations. This is consistent with published data reporting that chloroform is a strong cytotoxicant in cultures of L-929 fibrossarcoma cells (16). In the same way, eucalyptol induced cellular death when compared to the negative control at higher concentrations. Thus, it may be

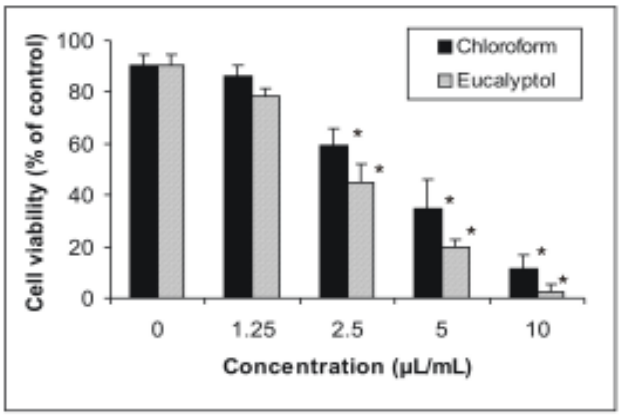

Figure 1. Effects of serial concentrations of chloroform and eucalyptol on trypan blue exclusion test. Results are expressed as the mean percentage of control (means ± SD). *$p<0.05$ compared to negative control (zero).

Table 1. Means (±SD) of DNA damage (tail moment) in mouse lymphoma cells exposed to the gutta-percha solvents.

Gutta-percha solvent	DNA damage (1.25 µL/mL)
Chloroform	0.74 ± 0.24
Eucalyptol	0.61 ± 0.20
Negative control[1]	0.61 ± 0.13
Positive control[2]	5.77 ± 0.78*

[1]Phosphate buffer solution (ph 7.4); [2]Methyl methanesulfonate (MMS) at 10 µL/mL; *$p<0.05$ compared to the negative control.

hypothesized that both solvents evaluated are able to bind to mouse lymphoma membrane and readily penetrate within the cells. With respect to the tested 1.25 µL/mL concentration, both solvents showed absence of cytotoxicity. Taken together, these findings suggest that, in terms of cellular injury, both gutta-percha solvents evaluated are potent cytotoxicants, and eucalytol presented more pronounced effects. It is important to stress that repeated exposure to cytotoxicants can result in chronic cell injury, compensatory cell proliferation, hyperplasia and ultimately tumor development (17).

Considering the strong evidence of a relationship between DNA damage and carcinogenesis, the alkaline version of the single cell gel (comet) assay used here is sensitive for a wide variety of DNA lesions. Among them are DNA strand breaks, alkali-labile sites lesions including abasic sites and incomplete repair sites (10). It shows clear benefits as concerns the applicability of almost all kind of cell types. In spite of its advantages, the limitations of this test system should be highlighted regarding its validity of detecting genotoxic effects in situations with elevated cytotoxic effects. Because cytotoxicity produces strand breaks that show up increased DNA migration, it is not recommended to perform the single cell gel (comet) assay on samples with more than 30% cytotoxicity (10). Therefore, the single cell gel (comet) assay was conducted for both gutta-percha solvents at the lowest concentration only. The results reported in this paper clearly demonstrated that chloroform did not induce DNA damage. Chloroform is classified as a Group 2B carcinogen by the International Agency for Research on Cancer (IARC) (18). This category is used to label agents that have an inadequate evidence of carcinogenicity in humans, but have sufficient evidence of carcinogenicity in experimental animals (19). In addition, eucalyptol did not induce DNA breakage as well. Previous studies have confirmed that eucalyptol is not considered a genotoxic carcinogen (20). A wide range of tests shall be performed to yield a more detailed assessment of the genotoxic potential of gutta-percha solvents.

The findings of this study support the conclusion that although chloroform and eucalyptol are strong cytotoxicants, it seems that they are not likely to increase the level of DNA damage on mammalian cells. Because cytotoxicity is considered a secondary mechanism in non-genotoxic carcinogenesis, continuous exposure to these compounds is of especial concern.

RESUMO

Clorofórmio e eucaliptol são amplamente utilizados na clínica odontológica como solventes de guta-percha. Entretanto, estes compostos podem representar um perigo à saúde humana, especialmente por causar danos ao aparelho genético e/ou induzir morte celular. Neste estudo, o potencial genotóxico e citotóxico associado à exposição ao clorofórmio e eucaliptol foram avaliados em células de linfoma murino *in vitro* pelo teste de células individualizadas (teste do cometa) e pelo teste do azul de tripan, respectivamente. Ambos os solventes de guta-percha provaram ser citotóxicos nos mesmos níveis em concentrações de 2,5, 5 e 10 µL/mL ($p<0.05$). Por outro lado, nenhum dos dois solventes induziu danos ao DNA. Em conclusão, esses resultados sugerem que ambos os compostos testados (clorofórmio e eucaliptol) são potentes citotoxinas, mas não representam um fator que aumenta o nível de danos no DNA em células de mamíferos.

ACKNOWLEDGEMENTS

This work was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico; Grant number: 141501/2002-2).

REFERENCES

1. Wennberg A, Orstavik D. Evaluation of alternatives to chloroform in endodontic practice. Endod Dent Traumatol 1989;5:234-237.
2. Wourns D, Campbell AD, Hicks ML, Pelleu GB. Alternative solvents to chloroform for gutta-percha removal. J Endod 1990;16:224-226.
3. Reuber MD. Carcinogenicity of chloroform. Environ Health Perspect 1979;31:171-182.
4. Hunter KR, Doblecki W, Pelleu GB. Halothane and eucalyptol as alternatives to chloroform for softening gutta-percha. J Endod 1991;17:310-312.
5. Squire RA. Ranking animal carcinogens: a proposed regulatory approach. Science 1981;214:877-880.
6. Kaplowitz GJ. Evaluation of the ability of essential oils to dissolve gutta-percha. J Endod 1991;17:448-449.
7. Hansen MG. Relative efficacy of solvents used in Endodontics. J Endod 1998;24:38-40.
8. Pecora JD, Spanó JCE, Barbin EL. *In vitro* study on the softening of gutta-percha cones in endodontic re-treatment. Braz Dent J 1993;4:43-47.
9. Auletta A, Ashby J. Workshop on the relationship between short-term information and carcinogenicity. Environ Mol Mutagen 1988;11:135-145.
10. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutag 2000;35:206-221.
11. 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.
12. McKelvey-Martin VJ, Green MH, Schmezer P, Pool-Zobel BL, De Meo MP, Collins A. The single cell gel electrophore-

sis assay (comet assay): a European review. Mutat Res 1993;288:47-63.
13. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendations for conducting the *in vivo* alkaline comet assay. Mutagenesis 2003;18:45-51.
14. Geurtsen W. Substances released from dental resin composites and glass ionomer cements. Eur J Oral Sci 1998;106:687-695.
15. Ribeiro DA, Marques MEA, Salvadori DMF. Lack of genotoxicity of formocresol, paramonochlorophenol and calcium hydroxide on mammalian cells by comet assay. J Endod 2004;30:593-596.
16. Vajrabhaya L, Suwannawong SK, Kamolroongwarakul R, Pewklieng L. Cytotoxicity evaluation of gutta-percha solvents: chloroform and GP-solvent (limonene). Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;98:756-759.
17. Mally A, Chipman JK. Non-genotoxic carcinogens: early effects on gap junctions, cell proliferation and apoptosis in the rat. Toxicology 2002;180:223-248.
18. IARC monographs on the evaluation of carcinogenic risk to humans. International Agency for Research on Cancer; Geneva, Switzerland. Suppl 7;1987. p.152-154.
19. NCI. Carcinogen bioassay for chloroform. National Cancer Institute. Bethesda, MD. National Technical Information Service No. PB2640018/AS; 1976.
20. U.S. Department of Health and Human Services. Public Health Service Fourth Annual Report on Carcinogens. PB85134663;1985.

Accepted October 13, 2005