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Radiopacifiers do not induce genetic damage in murine fibroblasts: an *in vitro* study

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Abstract

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Aim To evaluate whether several radiopacifiers are able to induce genetic damage in a laboratory cell culture study.

Methodology Murine fibroblasts were exposed to barium sulphate, bismuth oxide or zirconium oxide, at final concentrations ranging from 10 to 1 000 μg mL^{-1} for 1 h at 37 °C. The negative control group was treated with a vehicle control [phosphate buffered solution (PBS)] for 1 h at 37 °C and the positive control group was treated with hydrogen peroxide (at 10 μM) for 5 min on ice. Genotoxicity data were assessed by the single-cell gel (comet) assay.

Results All the tested compounds did not induce DNA breakage as depicted by the mean tail moment in all the concentrations analysed.

Conclusion Exposure to the tested radiopacifiers may not be a factor that increases the level of DNA lesions in mammalian cells as detected by a single-cell gel (comet) assay.

Keywords: barium sulphate, bismuth oxide, DNA damage, single cell gel (comet) assay, zirconium oxide

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Introduction

Some biomaterials do not possess sufficient radiopacity for clinical use and various chemical compounds, such as barium sulphate ($BaSO_4$), zirconium oxide or bismuth oxide may be added. However, several studies report that the presence of radiopacifiers can negatively influence the original properties of the material as well as promote noxious activities on the cellular level (Catelas *et al.* 1999, Ginebra *et al.* 2002, Mitchell *et al.* 2003).

Carcinogenesis is a multistage process, as a result of DNA damage and subsequent accumulation of a number of mutations in critical genes involved in the control of cell division, cell death and metastatic potential (Ribeiro *et al.* 2007). With increasing knowledge of these mechanisms, and the conclusion that most cases of cancer are preventable, efforts have focused on identifying the agents and exposures that cause the disease (Brendler-Schwaab *et al.* 2006). Genotoxicity tests can be defined as a laboratory and *in vitro* tests designed to detect compounds that induce genetic damage. Genotoxicity assays have gained widespread acceptance as an important and useful indicator of carcinogenicity (Dearfield & Moore 2005) as they are able to measure an initiating event in tumorigenesis and because of the reported high associations between positive responses in genotoxicity tests and human carcinogenicity (Fearon & Vogelstein 1990). For this reason, genotoxicity data are needed for the comprehensive risk assessment of dental compounds (Ribeiro 2008), particularly on radiopacifiers since few reports are available (Braz *et al.* 2008).

As a result, the goal of the present study was to evaluate if, and to what extent, $BaSO_4$, bismuth oxide and zirconium oxide are able to induce genetic damage *ex vivo*. For this purpose, the single-cell gel (comet) assay was applied in this setting.

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Materials and methods

Cell culture

Murine fibroblast cells (lineage 3T3-L1) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in a growth medium containing the following constituents: Dulbecco's modified Eagle'smedium (Invitrogen, Carlsbad, CA, USA) with 25 mmol L^{-1} glucose, 1 mmol L^{-1} pyruvate, 4.02 mmol L^{-1} L-alanyl-glutamine and 10% foetal calf serum (Sigma, St. Louis, MO,USA). Cells were cultured for 15 days prior to treatment with test substances. Confluent cells were detached with 0.15% trypsin (Invitrogen) for 5 min after which 2 mL complete medium was added and cells were centrifuged at 1 000 rpm (180*g*) for 5 min. Cell suspension was counted using a Neubauer chamber and seeded in 96-well microtitre plates (Corning, Acton, MA, USA) at a density of 1×10^4 cells per well (at a concentration of 1×10^6 mL^{-1}).

Cell treatment

The materials used were barium, bismuth oxide and zirconium oxide. All chemicals (powders) were purchased from one supplier (Reagen, Sao Paulo, SP, Brazil). To determine the significance of the concentration-related effect, increasing concentrations were tested ranging from 10 to 1 000 μg mL^{-1}. These concentrations were established in a pilot study (Braz *et al.* 2008). All the tested materials were dissolved in phosphate-buffered saline (PBS). Cells in culture were exposed to $BaSO_4$, bismuth oxide or zirconium oxide with different concentrations as described above for 1 h at 37 °C. The negative control group was treated with vehicle control (PBS) for 1 h at 37 °C as well. As for positive control group, cells were exposed to hydrogen peroxide (H_2O_2; Sigma-Aldrich) at 10 μM for 5 min on ice. Each treatment was tested thrice on three consecutive days. Therefore, a total of three wells were used for each individual treatment. After incubation, the cells were centrifuged at 1 000 rpm for 5 min, washed twice with a fresh medium and resuspended in the same medium.

Single-cell gel (comet) assay

The protocol used for a single-cell gel (comet) assay followed the guidelines described by Tice *et al.* (2000). Slides were prepared in duplicate per treatment. Thus, a volume of 10 μL of treated or control cells ($\sim 1 \times 10^4$ 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 a refrigerator, the coverslip was removed and slides immersed in lysis solution [2.5 M NaCl, 100 mM EDTA (Merck, Darmstadt, Germany); 10 mM Tris-HCl buffer pH 10 (Sigma); 1% sodium sarcosinate (Sigma); with 1% Triton X-100 (Sigma); and 10% DMSO (Merck)] for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer [0.3 mM NaOH; Merck and 1 mM EDTA (Merck) pH >13] for 20 min and electrophoresed for another 20 min, at 25 V (0.86 V cm^{-1}) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris–HCl (pH 7.5) for 15 min, fixed in absolute ethanol and stored at room temperature until analysis. All the steps described above were conducted in the dark to prevent additional DNA damage.

Throughout this study, several diluted and treated aliquots were tested for viability by trypan blue exclusion (Mckelvey-Martin *et al.* 1993).

Comet capture and analysis

A total of 50 randomly captured comets per treatment (25 cells from each slide) (Hartmann *et al.* 2003) were examined by one experienced observer who was blinded regarding the experimental groups, at 400× magnification using a fluorescence microscope (Olympus, Orangeburg, NY, USA.) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Haverhill, UK). For all experiments, two image-analysis parameters were evaluated: tail intensity (% migrated DNA) and tail moment. Tail moment was calculated by the image analysis system as the product of the tail length (DNA migration) and the fraction of DNA in the comet tail (% DNA in the tail). In none of the experiments was there a significant difference between these parameters (data not shown). Therefore tail moment was selected for the presentation of the results.

Statistical methods

The level of DNA damage (tail moment) was assessed using the Kruskal–Wallis test followed by the *post hoc* Dunn's test (SIGMASTAT software, version 1.0, Jandel Scientific, San Rafael, CA, USA). A P value <0.05 was considered statistically significant.

Results

The effect of the exposure of the test materials on DNA damage of murine fibroblasts is presented in Figs 1, 2 and 3. The assay detected a significant increase ($P = 0.02$) in tail moment for the positive control (H_2O_2-treated cells) compared with the negative controls. However, bismuth oxide did not promote DNA breakage at all concentrations tested. Similarly, zirconium oxide or $BaSO_4$ presented no detectable effect on genotoxicity at all the tested concentrations.

In all the treatment conditions, none of the three compounds increased cell mortality, as evidenced by constantly >75% of cells excluding trypan (data not shown).

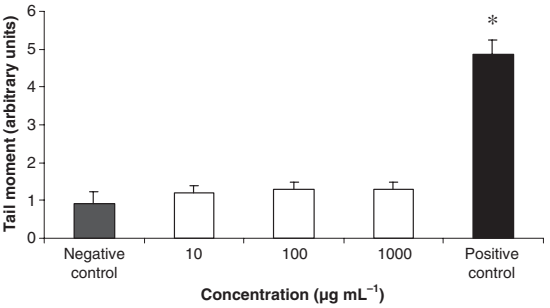

Figure 3 Genotoxic effects following exposure to serial concentrations of bismuth oxide in murine fibroblasts. Results are expressed as mean and SD. *$P < 0.05$ when compared to negative control ($n = 3$).

Discussion

The aim of this study was to evaluate the genotoxic damage induced by several radiopacifiers using a mammalian cell line *ex vivo*. The investigation was conducted utilizing the single-cell gel (comet) assay. This is rapid, simple and reliable biochemical technique for evaluating DNA damage in mammalian cells (Tice *et al.* 2000). The basic principle of the single-cell gel (comet) assay is the migration of DNA fragments in an agarose matrix under electrophoresis. When viewed using 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 (Singh *et al.* 1988). Recent studies have demonstrated that the single-cell gel (comet) assay is a suitable tool to investigate genotoxicity of compounds used in clinical practice (Ribeiro *et al.* 2004, 2006, Braz *et al.* 2006).

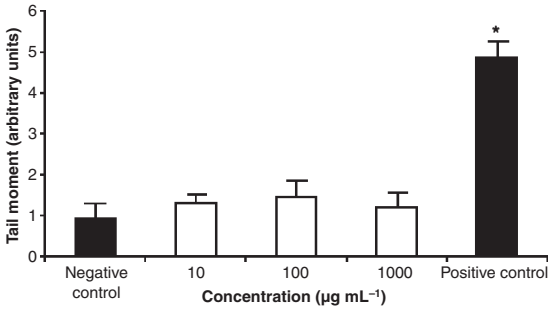

Figure 1 Genotoxic effects following exposure to serial concentrations of barium sulphate in murine fibroblasts. Results are expressed as mean and SD. *$P < 0.05$ when compared to negative control ($n = 3$).

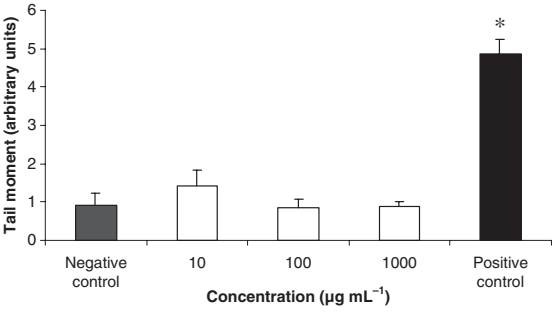

Figure 2 Genotoxic effects following exposure to serial concentrations of zirconium oxide in murine fibroblasts. Results are expressed as mean and SD. *$P < 0.05$ when compared to negative control ($n = 3$).

The alkaline version of the single-cell gel (comet) assay used in the present study is sensitive for a variety of DNA lesions. Amongst them are single- and double strand breaks, oxidative DNA base damage, alkali-labile sites including abasic and incomplete repair sites and DNA–DNA/DNA–protein/DNA–drug cross-linking in any eukaryotic cell (Tice *et al.* 2000). Tail moment is a virtual measure calculated by the computerized image analysis system considering both the length of DNA migration in the comet tail and the tail intensity. This parameter is one of the best indices of induced DNA damage amongst the various parameters calculated by this method (Tice *et al.* 2000). On the basis of tail moment data, the results of this study revealed that the alkaline single-cell gel (comet) assay in the experimental conditions used did not detect the presence of DNA damage after a treatment for bismuth oxide, zirconium oxide or $BaSO_4$ at all concentrations tested using murine fibroblast cells. Furthermore, no concentration–effect relationship for

all materials tested was found. It is important to emphasize that no single test is capable of detecting all genotoxic agents. Thus, for a more detailed judgment on the genotoxic potential of radiopacifiers, a battery of tests is advisable.

Accumulating evidence suggests that although some agents, notably $BaSO_4$ and zirconium dioxide, optimize the radiopaque properties of materials, their biocompatibility properties have been questioned. For example, a recent study has provided an evidence that $BaSO_4$ particles enhance marcophage–osteoclast differentiation, which contributes to bone resorption, ultimately resulting in aseptic loosening of cemented hip prostheses (Mitchell *et al.* 2003). Therefore, there are some concerns about the biological activity of the radiopaque agents added to these materials (Sabokbar *et al.* 1997). The present data revealed that neither $BaSO_4$ nor zirconium oxide caused DNA-damage at all concentrations tested in immortalized murine fibroblasts. Furthermore, a recent study conduced has reported that these radiopacifiers were not able to induce genetic damage in ordinary human lymphocytes *ex vivo* (Braz *et al.* 2008). However, the genotoxicity induced by these radiopacifiers may be modulated in combination with other DNA-damaging agents that are present in the environment. In this way, further studies are required to analyse this issue.

In this current investigation, hydrogen peroxide was used as a positive control. Hydrogen peroxide is a molecule that passes through the cell membrane and is transformed in to hydroxyl radicals by a non-enzymatic process in the presence of metal ions (Fe^{2+} or Cu^{2+}) occurring in the cytoplasm, known as the Haber–Weiss or Fenton reaction. Hydroxyl radicals can induce single-strand breaks, double-strand breaks, alkali-labile sites and various species of oxidized purines and pyrimidines (Joenge 1989). In the present study, a 10-μM H_2O_2 solution was used, which induced a high level of DNA damage without an associated higher cell mortality.

In the present study, using the single-cell gel (comet) assay, comets without clearly identifiable heads were excluded from the image analysis. Although it should be emphasized that it is still not completely understood what these events 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 radiopacifiers rather than primary DNA-damage following a direct interaction between DNA and a genotoxic agent (Ribeiro *et al.* 2005).

Conclusion

All radiopacifiers tested did not induce DNA damage *ex vivo*. Since DNA damage is an important step in events leading from carcinogen exposure to cancer, the results of the present study represent a potential alert to the correct evaluation of the potential health risks associated with exposure to these compounds.

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