J Mater Sci: Mater Med (2008) 19:601–605
DOI 10.1007/s10856-007-3000-2

Genotoxicity in primary human peripheral lymphocytes after exposure to radiopacifiers in vitro

Mariana Gobbo Braz · Joao Paulo de Castro Marcondes · Mariza Akemi Matsumoto · Marco Antonio Hungaro Duarte · Daisy Maria Favero Salvadori · Daniel Araki Ribeiro

Received: 17 September 2006 / Accepted: 22 February 2007 / Published online: 10 July 2007

Abstract Taking into consideration that DNA damage plays an important role in carcinogenesis, the purpose of this study was to evaluate whether some radiopacifiers widely used in clinical practice are able to induce genetic damage in primary human cells in vitro. Human peripheral lymphocytes obtained from 10 healthy volunteers were exposed to barium sulphate ($BaSO_4$), zirconium oxide (ZnO_2) and bismuth oxide (Bi_2O_3) at final concentrations ranging from 1 to 1000 μg/mL for 1 h at 37 °C. The negative control group was treated with vehicle control (phosphate buffer solution) for 1 h at 37 °C and the positive control group was treated with hydrogen peroxide (at 100 μM) for 5 min on ice. Results were analyzed by the Friedman non-parametric test. The results pointed all compounds tested out did not induce DNA breakage in human peripheral lymphocytes as depicted by the mean tail moment and tail intensity in all concentrations tested. In summary, our results indicate that exposure to these radiopacifiers may not be a factor that increases the level of DNA lesions in human peripheral lymphocytes as detected by single cell gel (comet) assay.

M. G. Braz · J. P. de Castro Marcondes · D. M. F. Salvadori
Department of Pathology, Botucatu Medical School, São Paulo State University, UNESP, Botucatu, SP, Brazil

M. A. Matsumoto · M. A. H. Duarte
Department of Dental Clinics, University of Sacred Heart, USC, Bauru, SP, Brazil

D. A. Ribeiro (✉)
Department of Health Sciences, Federal University of Sao Paulo, UNIFESP, Av. Ana Costa, 95, Vila Mathias, Santos, SP 11060-001, Brazil
e-mail: daribeiro@unifesp.br

Introduction

Nowadays, several biomaterials have been used in clinical practice. However, there are some problems associated with the use of these compounds, mainly related to radiopacity that is insufficient to adequately observe the perfect location of these ones. To overcome these drawbacks, radiopacity is introduced by particles of some chemical compounds, such as barium sulphate ($BaSO_4$), zirconium oxide (ZnO_2) and bismuth oxide (Bi_2O_3). Nevertheless, several studies report that the presence of radiopacifiers can negatively influence the original properties of the compound as well as to promote noxious actives on biological cellular systems [1–3].

Carcinogenesis is a multi-step process, which is characterized by genetic, epigenetic, and phenotypic changes [4]. Such changes involve genetic damage, mutation in critical genes related to the control of cell division, cell death and metastatic potential, and activation of signalizing or metabolic pathways that give the cells favorable growth and survival characteristics [5]. With increasing of 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 cancer [6]. Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds which induce genetic damage including DNA damage, gene mutation, chromosomal breakage, altered DNA repair capacity and cellular transformation. In the last decades, genotoxicity assays have gained widespread acceptance as an important and useful indicator of carcinogenicity [7]. For this reason, genotoxicity data are needed for the comprehensive risk assessment of radiopacifiers, particularly because there are no previous reports.

As a result and because of inappropriate evidence, the goal of the present study was to evaluate genotoxic effects of barium sulphate ($BaSO_4$), zirconium oxide (ZnO_2) and bismuth oxide (Bi_2O_3) in human peripheral lymphocytes in vitro. The most frequently cells used for in vitro studies when screening for the potential genotoxicity of chemicals are Chinese hamster ovary (CHO) cells or L5178 Y mouse lymphoma cells. However, there are some shortcomings associated with cell lines. For example, the chromosomal material has usually undergone extensive rearrangement, in which some key genes involved in affecting the viability of cells sustaining DNA damage such as *TP53*, may be mutated [8]. Human lymphocytes have the advantage of being karyotypically normal human cells as well as are good indicators of the systemic burden by exposure factors [9]. In this regard, we chose the human lymphocytes in this current study.

To evaluate the magnitude of DNA damage, the single cell gel (comet) assay was applied in this setting. This is rapid, simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells [10]. 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 [11]. Our own recent studies have demonstrated that the single cell gel (comet) assay is a suitable tool to investigate genotoxicity of compounds used in clinical practice [12, 13].

Materials and methods

Subjects

A total of 10 healthy donors were used in this study. Five donors were female being 23–31 years of age and five were male donors being 21–27 years-old. Each person was interviewed about possible confounding factors and was excluded from this study when there was a history of smoking or cancer, previous radio- or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays during the last 6 months, intensive sportive activities during the last week and high alcohol consumption. All blood donors gave informed consent to participate in this study and the study was approved by the Ethical Committee for Human Research, Botucatu Medical School, UNESP, SP, Brazil.

Cell preparation

Heparanized blood samples were obtained by venous puncture. A total of 2 mL of peripheral blood was collected from all donors. The lymphocytes were isolated on Ficoll-Paque gradients according to the technique described by Fenech [14] with slight modifications. Namely, samples from 2 mL of peripheral blood were added to 2 mL of phosphate-buffered saline (PBS) and layered over 3 mL using Ficoll-Paque Plus (Amersham, Sweden). This was centrifuged at 1100*g* for 30 min. The lymphocytes were taken and washed in 4 mL of PBS at 400 g for 15 min. The cell pellet (~500 μl) containing ~1 × 1000000 cells per mL was transferred to a micro tube and destined to cell treatment.

Cell treatment

The materials used were barium sulphate ($BaSO_4$), zirconium oxide (ZnO_2) and bismuth oxide (Bi_2O_3). All chemicals were purchased from Reagen, SP, Brazil. To determine the concentration-related effect significance, we were able to test in increasing final concentrations ranging from 1–1000 μg/mL for 1 h at 37 °C. These concentrations were established in a previous pilot study conducted in our laboratory. All materials tested were dissolved in PBS. The negative control group was treated with vehicle control (PBS) for 1 h at 37 °C as well. For positive control group, lymphocytes were exposed to hydrogen peroxide (H_2O_2, Sigma Aldrich, St. Louis, MO, USA) at 100 μM during 5 min on ice.

Single cell gel (comet) assay

The protocol used for single cell gel (comet) assay followed the guidelines purposed by Tice et al. [10]. Slides were prepared in duplicate per treatment. Thus, a volume of 10 μl of treated or control cells (~1×10^4 cells) was 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 slides immersed to lysis solution (2.5 M NaCl, 100 mM EDTA—Merck, St Louis, USA; 10 mM Tris-HCl buffer pH = 10—Sigma Aldrich, USA; 1% sodium sarcosinate—Sigma Aldrich, USA; with 1% Triton X-100—Sigma Aldrich, USA; and 10% DMSO—Merck St. Louis, USA) for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer (0.3 mM NaOH, Merck USA; and 1 mM EDTA, Merck, 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) for 15 min, fixed in absolute ethanol and stored at room temperature until analysis. All of the steps described above were conducted in the dark to prevent additional DNA damage.

Throughout this study, some diluted and treated aliquots were tested for viability by trypan blue exclusion [15], and constantly >75% of cells excluded trypan.

Comet capture and analysis

A total of 50 randomly captured comets per treatment (25 cells from each slide) [16] were examined blindly by one expertise observer at 400× magnification using a fluorescence microscope (Olympus) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Haverhill, UK). For all experiments, we evaluated two image analysis parameters: 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).

Statistical methods

Parameter from the comet assay (tail moment) was assessed by the Friedman non-parametric test [17], using SigmaStat software, version 1.0 (Jadel Scientific, Rafael, CA, USA). A p value less than 0.05 was considered statistically significant.

Results

Exposure of human lymphocytes with all radiopacifiers did not induce genotoxic effects in the single cell gel (comet) assay for all concentrations tested. Furthermore, all treatments did not indicate any concentration-effect relationship. These results are summarized in Figs. 1, 2 and 3. In all treatment conditions, none of the three compounds increased cell mortality, in which constantly >75% of cells excluded trypan (data not shown). For comparison, the comet assay was able to detect the significant increase in tail moment of positive control (H_2O_2) when compared to negative control at high levels of cellular viability. Figure 4a–c shows undamaged lymphocytes exposed to PBS (negative control), barium sulphate ($BaSO_4$), and positive control, respectively.

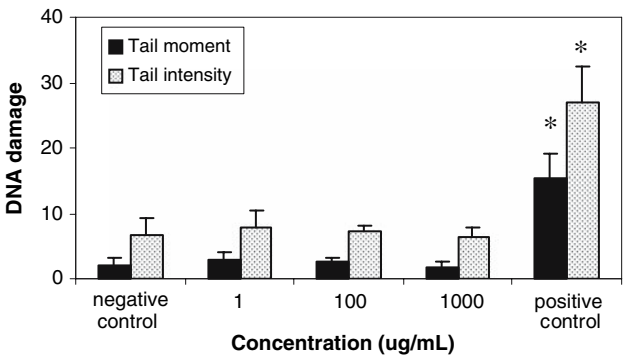

Fig. 1 Genotoxic effects following exposure to serial concentrations of barium sulphate. Results are expressed as mean and standard deviation. *$p < 0.05$ when compared to negative control

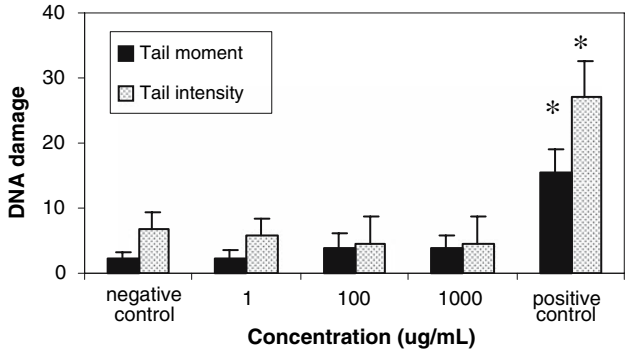

Fig. 2 Genotoxic effects following exposure to serial concentrations of zirconium oxide. Results are expressed as mean and standard deviation. *$p < 0.05$ when compared to negative control

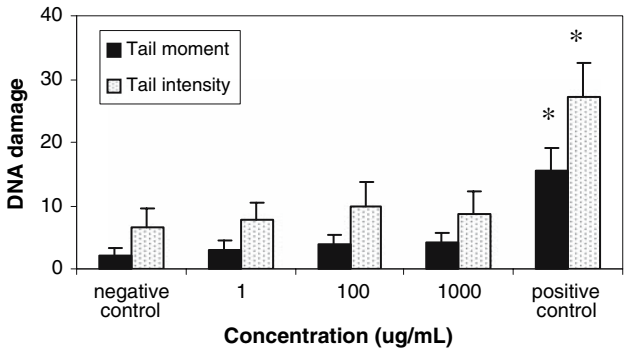

Fig. 3 Genotoxic effects following exposure to serial concentrations of bismuth oxide. Results are expressed as mean and standard deviation. *$p < 0.05$ when compared to negative control

Discussion

The aim of this study was to evaluate the genotoxic damage induced by some radiopacifiers on human peripheral lymphocytes in vitro. The investigation was conducted utilizing the single cell gel (comet) assay. To best of our knowledge, the approach has not been demonstrated so far.

The alkaline version of the single cell gel (comet) assay used here is sensitive for a wide variety of DNA lesions. Among 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 [10]. Tail moment is a virtual measure calculated by the computerized

Fig. 4 Representative comet images from human lymphocyte: (**a**) negative control; (**b**) barium sulphate treated-cell; (**c**) and hydrogen peroxide-treated cell (positive control). (DNA was stained with ethidium bromide; ×40 magnification)

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 among the various parameters calculated by this method. On the basis of tail moment and tail intensity data, the results of this study pointed out that the alkaline single cell gel (comet) assay in the experimental conditions used, did not detect the presence of DNA damage after a treatment by bismuth oxide in all concentrations tested. 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 feasible.

Accumulating evidence suggests that although some agents, notably barium sulphate and zirconium dioxide optimize the radiopaque properties of materials, their biocompatibility properties have been questioned at high levels. For example, a recent study has provided evidence that $BaSO_4$ particles enhance marcophage-osteoclast differentiation, which contributes to bone resorption, ultimately resulting in aseptic loosening of the prosthesis [3]. Therefore, there are some concerns about the biological activity of the radiopaque agents added to these materials [18]. Our data revealed that neither barium sulfate nor zirconium oxide caused DNA-damaging in all concentrations tested. 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 analyze this issue.

In this current investigation, we were able to use the hydrogen peroxide as positive control. Hydrogen peroxide is a molecule that easily goes through the cell membrane and is transformed in 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 [19]. In studies performed with human lymphocytes, the hydrogen peroxide concentrations most frequently used to induce DNA damage range from 10 μM to 280 μM [20, 21] In the present study, we used a 100 μM H_2O_2 solution, which induced a high DNA damage level without a higher cell mortality associated.

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 hedgehogs 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 [22].

Conclusively, the present study indicates that all radiopacifiers tested were not able to induce DNA damage in human lymphocytes in vitro. Since DNA damage is an important step in events leading from carcinogen exposure to cancer, the results of present study represent a potential alert to the correct evaluation of the potential health risks associated with exposure to these compounds.

Acknowledgements We are thankful to Maria Luiza Falaguera Ardanaz for her technical assistance. This work was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo).

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