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Genotoxicity and oxidative stress in gasoline station attendants

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ABSTRACT

We evaluated genotoxic effects of exposure to low levels of benzene, a class I human carcinogen, among gasoline station attendants (GSA). Oxidative stress and the protective effects of antioxidants on DNA damage were also analyzed. Although exposures were below ACGIH (American Conference of Governmental Industrial Hygienists) limits, the GSA group presented higher DNA damage indices and micronucleus frequencies, increased oxidative protein damage, and decreased antioxidant capacity relative to the control group. Duration of benzene exposure was correlated with DNA and protein damage. The biomarkers evaluated in this work may provide early signals of damage in subjects occupationally exposed to benzene.

1. Introduction

Benzene, a hazardous component of gasoline, is a genotoxic class I human carcinogen [1–4]. Benzene exposure has been strictly regulated [5]; its use in workplaces is declining, reducing occupational exposure and associated health hazards [6,7]. Concern remains with regards to prolonged exposure to low levels of benzene in occupational and environmental settings [7].

The mechanisms of benzene toxicity remain elusive [8]. Reactive oxygen species (superoxide anion, hydrogen peroxide, hydroxyl radical) resulting from benzene metabolism [3,9,10] may damage biomolecules [5,6], and may lead to DNA strand breaks, micronuclei, and chromosomal aberrations [6,11]. Thus, the genotoxic effects of benzene may be associated with oxidative DNA damage [11,12].

Biological monitoring is fundamental for evaluation of risks to human health due to exposure to chemical substances [7]. In Brazil, biological monitoring of occupational benzene exposure is performed by quantification of the urinary metabolite *t*,*t*-muconic acid [13], formed *via* cytochrome P450-catalyzed oxidation [9,14].

Evaluation of biomarkers of susceptibility or effect are also important tools [15]. The comet and micronucleus assays are widely used in biomonitoring studies to evaluate genotoxicity and DNA damage [4,12,16–19]. Oxidative damage biomarkers can also supply information about early biological effects and eventual health risks [3].

We have investigated genotoxicity caused by low-level exposure to benzene among gasoline station attendants. Comet and micronucleus assays were conducted to evaluate genetic damage. The involvement of oxidative stress was assessed through biomarkers of protein oxidation and nitration. Possible protective effects of endogenous and exogenous antioxidants were also analyzed.

2. Materials and methods

2.1. Subjects

Initially, 105 male subjects were recruited, including 70 exposed workers and 35 non-exposed individuals. All participants were interviewed about aspects of general health, lifestyle, smoking status, and history of exposure. Individuals were excluded based on any diagnosed diseases, taking medication or antioxidant vitamins at the time of the study, smoking, and any subjects who had failed to provide the required samples of personal air, urine, blood, and buccal cells. Individuals who had worked

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in their current job position for less than 6 months were also excluded. Based on these exclusion criteria, 71 men were enrolled. The exposed group comprised 43 gasoline station attendants (GSA). The control group consisted of 28 subjects with no history of occupational exposure to benzene or other xenobiotics. The average ages of the GSA and control groups were 33.9 ± 1.5 and 30.4 ± 1.8 years, respectively. No significant difference between GSA and control group was found related to age. The mean exposure time in the GSA group was 9.7 ± 1.1 years (range 0.5–32 years). This study was approved by the Research Ethics Committee of the Federal University of Rio Grande do Sul/RS (No. 21728/11) and informed consent was obtained from all participants prior to their enrollment in the study.

2.2. Sampling

Sampling was conducted at the end of the work shift, after 3–4 consecutive days of exposure. Individual air samples were collected in the breathing zone of study subjects during the daily work shift, for approximately 8 h. After air sampling, the samples were capped, transported to the lab and stored at −20 °C until analysis. Urine samples were collected and stored in polyethylene bottles at −80 °C until *t,t*-muconic acid analysis. Blood venous samples were collected by venipuncture using vacuum tubes. EDTA-blood tube was collected and centrifuged at 1500 × *g* for 10 min at 4 °C. Aliquots of EDTA-plasma were stored at −80 °C until analysis of the protein damage biomarkers, α-tocopherol, retinol, lycopene, and β-carotene. The remaining erythrocytes were used to measure reduced glutathione content. A blood-heparin tube was collected for comet assay analysis. To avoid any damage associated with storage, the samples were processed immediately. The remaining heparin-blood was stored at −80 °C for antioxidant enzymes analysis. Serum was obtained by centrifugation at 1500 × *g* for 10 min and stored at −80 °C until measurement of nitric oxide and vitamin C levels. Additionally, buccal cells were collected for the micronucleus assay.

2.3. Benzene exposure

Airborne benzene was collected using passive samplers (SKC 575-002®). Benzene was determined in samples after desorption with dichloromethane followed by gas chromatography with flame ionization detection (GC-FID) analysis. Chromatographic separation was achieved using an Innowax GC column (25 m, 0.2 mm, 0.4 μm), with the following chromatographic conditions: 99.99% purity H_2 was used as carrier gas, pressure 10,055 psi, initial column temperature 40 °C, increased at 4 °C min^{-1} to 53 °C, followed by an oven ramp rate of 40 °C min^{-1} to 200 °C. FID detector temperature was kept at 250 °C. The results were expressed as μg m^{-3}.

2.4. Urinary t,t-muconic acid (t,t-MA)

Urinary *t,t*-MA was measured by solid-phase extraction followed by high performance liquid chromatography and UV detection (HPLC-UV), according to the method previously described [20], with some modifications. The *t,t*-MA levels were expressed as μg g^{-1} creatinine.

2.5. Creatinine concentration

Urine samples were collected and refrigerated at 4 °C until further analysis. Creatinine concentration was measured by spectrophotometry [21], utilizing commercial kits (Doles Reagents, Goiânia, GO, Brazil).

2.6. Comet assay

A standard protocol was adopted for comet assay preparation and analysis [22,23]. Slides were prepared by mixing 5 μL whole blood and 95 μL low-melting-point agarose (0.75%). The mixture was poured into a frosted microscope slide coated with normal-melting-point agarose (1.5%). After solidification, the coverslip was removed and the slides were placed in lysis solution (NaCl 2.5 M, ethylenediaminetetraacetic acid (EDTA) 100 mM, Tris 10 mM, pH 10.0–10.5, with freshly prepared Triton X-100 1% and dimethylsulfoxide 10%) for a minimum of 1 h and a maximum of 5 days. Subsequently, the slides were incubated in freshly made alkaline solution (NaOH 300 mM and EDTA 1 mM, pH 12.6) for 10 min. Separation of DNA fragments was obtained by electrophoresis for 20 min at 25 V (0.9 V cm^{1}) and 300 mA and then the solution was neutralized with Tris 0.4 M (pH 7.5). Finally, DNA was stained with silver nitrate, and the slides were coded for blind analysis. The electrophoresis procedures and the efficiency for each electrophoresis run were checked using negative and positive reference controls. The negative reference control was whole blood and the positive reference control consisted of whole blood mixed with methyl methanesulfonate at a final concentration of 8×10^{-5} M. This mixture was incubated at 37 °C for 2 h. Images of 100 randomly selected cells (50 cells from each of two replicated slides) were analyzed from each sample. Each electrophoresis run was considered valid only if the negative and positive controls yielded the expected results. DNA damages were visually scored according to tail size into five classes, from no tail (0) to maximal (4) tail length, resulting in a single DNA damage score (DNA damage index – DNA DI). Therefore, the DNA damage score could range from 0 (all cells with no tail, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4). All analyses performed in this study were considered valid when DNA DI values in positive and negative controls were 380–400 and 0–4, respectively.

2.7. Micronucleus (MN) assay

For the micronucleus assay, buccal cells were collected at the end of the work shift. Subjects were asked to wash their oral cavities with water before sampling. Small brushes were used to collect samples from buccal mucosa. Cells were fixed with acetic acid: methanol (75:25, v/v), transferred onto clean microscope slides in duplicates and stained with Giemsa 5%. The criterion of scoring cells with MN was as described in the literature [24]. One thousand cells were counted for each sample. Results were expressed as the micronucleus frequency per 1000 cells.

2.8. Protein carbonyl (PCO) assay

PCO were determined using a noncompetitive ELISA method [25], with some modifications. Total protein concentration in plasma was measured by the Bradford method using bovine serum albumin as standard. PCO levels were determined as follows: plasma samples were diluted with PBS buffer to a normalized concentration of 4 mg protein mL^{-1} and then samples were derivatized with 2,4-dinitro-phenylhydrazine (DNPH) and incubated in Maxisorb multiwallplates (Nunc Immuno 96 Microwell™ Maxisorp) overnight at 4 °C in the dark. Protein carbonyls were detected using a dinitrophenyl rabbit IgG-antiserum (Sigma) as the primary antibody and a monoclonal anti-rabbit immunoglobulin G peroxidase conjugate (Sigma) as the secondary antibody. Color development was performed with *o*-phenylenediamine and H_2O_2 and the reaction was stopped with H_2SO_4 after 15 min incubation at 37 °C. The absorbance was measured using a microplate reader with a detection wavelength of 492 nm. Each sample was analyzed in triplicate. Plasma protein carbonyl concentration was expressed as nmol mg^{-1} protein.

2.9. 3-Nitrotyrosine (3-NT) assay

3-NT plasma levels were assessed according to Weber et al. [26]. Total protein concentration in plasma was measured by the Bradford method and the samples were diluted to 2 mg protein mL^{-1}. The diluted samples were incubated in Maxisorb multiwallplates (Nunc Immuno 96 Microwell™ Maxisorp) overnight at 4 °C in the dark. A noncompetitive ELISA method was used to measure 3-NT levels. Polyclonal anti-nitrotyrosine (Millipore) and monoclonal goat anti-rabbit IgG, HRP-conjugate (Millipore) were used as primary and secondary antibodies, respectively. After color development, the reaction was stopped and the absorbance was measured at 492 nm in an ELISA microplate reader. The samples were assayed in triplicate. The 3-NT content in plasma was expressed as pmol mg^{-1} protein.

2.10. Nitric oxide (NO)

Serum NO levels, indicated by nitrites/nitrates (NO_x) concentration, were measured in the Cobas Mira automated analyzer, by a modified Griess method as described by Tatsch et al. [27]. The contents of NO were expressed as μmol L^{-1}.

2.11. Erythrocyte reduced glutathione (GSH)

GSH levels were measured by spectrophotometry at 412 nm, as previously described in the literature [28]. The erythrocytes were hemolyzed by Triton X-100, and precipitated with 20% trichloroacetic acid (w/v). After centrifugation, the supernatant aliquots were reacted with 10 mM of 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB). The GSH content was expressed as μmol mL^{-1} of erythrocytes.

2.12. Antioxidant enzymes

Enzyme assays were performed in 96-well microplates and measured by spectrophotometry in a microplate reader (SpectraMax M2, Molecular Devices). Superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities were determined as previously described [29–31]. Activities were expressed as units per mg protein.

2.13. Exogenous antioxidants

Serum vitamin C levels were quantified by HPLC with UV detector (HPLC-UV), according to a method developed by our group [32]. Serum samples were deproteinized with 10% perchloric acid (v/v) and the supernatants were measured at 245 nm. Vitamin C concentrations were expressed as mg L^{-1}.

Simultaneous quantification of α-tocopherol, retinol, lycopene, and β-carotene was performed according to a method developed in our laboratory [33]. Plasma samples were extracted with ethanol:n-butanol solution (50:50, v/v) and supernatants were injected into the HPLC system. Samples had their absorption monitored at 450 nm for the quantification of lycopene and β-carotene. Fluorescence at two different excitation and emission wavelengths was monitored to quantify retinol (340 and 520 nm, exc./em.) and α-tocopherol (298 and 328 nm, exc./em.). Results were expressed as μmol L^{-1}.