Evaluation of genotoxicity and oxidative damage in painters exposed to low levels of toluene

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ABSTRACT

Toluene is an organic solvent used in numerous processes and products, including industrial paints. Toluene neurotoxicity and reproductive toxicity are well recognized; however, its genotoxicity is still under discussion, and toluene is not classified as a carcinogenic solvent. Using the comet assay and the micronucleus test for detection of possible genotoxic effects of toluene, we monitored industrial painters from Rio Grande do Sul, Brazil. The putative involvement of oxidative stress in genetic damage and the influences of age, smoking, alcohol consumption, and exposure time were also assessed. Although all biomarkers of toluene exposure were below the biological exposure limits, painters presented significantly higher DNA damage (comet assay) than the control group; however, in the micronucleus assay, no significant difference was observed. Painters also showed alterations in hepatic enzymes and albumin levels, as well as oxidative damage, suggesting the involvement of oxidative stress. According to multiple linear regression analysis, blood toluene levels may account for the increased DNA damage in painters. In summary, this study showed that low levels of toluene exposure can cause genetic damage, and this is related to oxidative stress, age, and time of exposure.

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1. Introduction

Biological monitoring of exposure to toxic chemicals in the workplace is a fundamental tool to evaluate human health risks and to improve occupational safety [1,2]. Toluene is an organic solvent used in numerous products, including industrial paints, adhesives, coatings, inks, and cleaning products [3]. Although toluene use in workplaces has decreased, it remains the most commonly used organic solvent in manufacturing the products listed above [4]. The highest levels of toluene exposure are seen in the painting, printing, automotive, and shoemaking industries [5].

Several biomarkers are available to assess internal exposure to toluene [3]. In the occupational setting, exposure has been monitored through measurement of metabolites in urine and the parent compound in blood [6]. The ideal indicator would accurately reflect the target-tissue concentration of the toxicologically active species (parent or metabolite) in a time-frame related to the onset of the toxic effect (acute, subchronic, or chronic) [5].

Toluene neurotoxicity and reproductive toxicity are accepted facts [7–9]. However, its genotoxicity is still under discussion and it is not classified as a carcinogenic solvent [8,9]. Epidemiological studies have reported significant increases of respiratory tract cancer, lung cancer, kidney cancer, urinary bladder cancer, and leukemia in printing workers [10–12]. More recent studies have provided evidence of genotoxic effects in painters and shoe manufacturers, also included in the “high risk of cancer” occupational groups [1,13–15].

The evaluation of genetic damage in populations exposed to xenobiotics in workplaces, using genotoxicity biomarkers, can play an important role in predicting health risk and detecting human genotoxic exposure and its effects [1,16]. Many methods are being used for detecting early biological effects of DNA-damaging agents in occupational settings [17].
The comet assay is a reliable method for monitoring and evaluating DNA damage in humans. It is a rapid and sensitive tool to analyze chemically induced DNA damage, detecting strand breaks, alkali-labile sites, DNA crosslinking, and incomplete excision repair sites [18,19]. The micronucleus test (MNT) is another cytogenetic test used to assess occupational exposure to toxic agents [14,20,21]. This test provides a measurement of events related to carcinogenesis, such as chromosome breakage, chromosome loss, or interference with the mitotic apparatus [22]. The buccal cell MNT is a non-invasive technique that has become an attractive candidate for biomonitoring human populations [23]. Oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents, such as organic solvents, entering the body via inhalation and ingestion [24].

One pathway of tissue biotransformation leads to the formation of toluene epoxides, which may generate reactive oxygen species (ROS) and can cause oxidative stress and DNA damage [25]. DNA oxidation has potential genotoxic consequences [26] and is implicated in the pathogenesis of several diseases [27], including cancer and neurodegenerative disorders [28,29].

The aim of this study was to evaluate genetic damage caused by low-level exposure to toluene. We biomonitored painters from an industry in Rio Grande do Sul, Brazil. The comet assay and micronucleus test were used for detecting the possible genotoxic effects of this solvent. Lipid peroxidation was assessed by malondialdehyde (MDA) concentration and protein oxidation was evaluated by the protein carbonyl (PCO) assay. Aiming to determine the possible involvement of reactive oxygen species in the induction of genotoxicity, ischemia-modified albumin (IMA) and albumin (ALB) levels were also measured. The possible influences of confounding factors such as age, smoking, alcohol consumption and exposure time on genotoxic effects were also analyzed.

2. Materials and methods

2.1. Subjects

Sixty-one subjects participated in this study. The exposed group was comprised of 34 male industrial painters, from Rio Grande do Sul, Brazil, occupationally exposed to toluene, the main component of paints used by them. The control group consisted of 27 subjects with no history of occupational exposure. All subjects answered an investigator-administered questionnaire about general health, lifestyle (smoking and alcohol drinking habits) and time of occupational exposure. The study was approved by the Committee of Ethics in Research of the Federal University of Santa Maria/RS and informed consent was obtained from all participants, according to the guidelines of the local committee.

2.2. Sample collection

Urine, blood, and buccal cells samples were obtained at the end of the work shift on the last day of the work week. Urine samples (50 mL) were collected for determination of toluene metabolites, creatinine, and cotinine. The samples were stored in polyethylene bottles and refrigerated at 4 °C until further analysis (within 10 days of sampling). Two EDTA vacuum blood tubes were collected. The first tube was used for toluene quantification; after collection, it was immediately sealed and kept at −80 °C to avoid losses, because toluene is volatile. Another EDTA vacuum blood collection tube was immediately centrifuged at 1500 g for 10 min at 4 °C, and the plasma was used to quantify MDA and PCO levels. A vacuum blood collection tube containing heparin was used for the comet assay. It was kept on ice and processed as soon as possible, to avoid any damage associated with storage. Another vacuum blood collection tube without anticoagulant was centrifuged at 1500 g for 10 min at room temperature. The serum obtained was used for IMA, albumin, and hepatic enzymes determination.

2.3. Blood toluene levels

In 10 mL headspace vials, blood samples (1 mL) were added together with 25% NaCl(w/v), 4 mL, and 20 μg mL⁻¹ nitrobenzene (internal standard, IS; 100 μL). After vortexing (10 s), NaCl (0.2 g) was added, to achieve the salting-out effect. Toluene and IS were extracted by solid-phase microextraction (SPME). The samples were extracted using a Carboxen/PDMS fiber, obtained from Supelco® (Bellefonte, USA), for 10 min at 50 °C, at a mixing velocity of 250 rpm. The analytes were desorbed at 250 °C for 3 min. Gas-chromatographic separation was performed using a CP 3800 gas chromatograph (Varian, Middlesburg, The Netherlands) with an OV-1 column (30 m, 0.32 mm, 1 μm), from Ohio Valley (Marietta, USA). Carrier gas (helium) flow rate was 1.9 cm⁻¹ min⁻¹. The initial oven temperature was 100 °C, maintained for 2 min, and then increased 15 °C min⁻¹ until 180 °C, which was maintained for 0.7 min. The total running time was 8 min. Detection was made by a flame ionization detector (FID), kept at 250 °C. The retention times were 2 and 4.5 min for toluene and IS, respectively. The detection and quantification limits of the method were 0.02 and 0.05 mg L⁻¹, respectively.

2.4. Determination of urinary toluene metabolites

Quantification of urinary levels of hippuric acid (HA), the main toluene metabolite, was performed according to a HPLC-UV method previously standardized in our laboratory [30].

Urinary ortho-cresol (o-C), another toluene metabolite, was also quantified. In order to determine o-C concentration, a urine sample (1 mL) was hydrolyzed with conc. HCl (100 μL) and placed in boiling water bath for 40 min. After cooling and addition of nitrobenzene methanolic solution (internal standard), 2 μg mL⁻¹, 100 μL, the sample pH was adjusted with the addition of 50% NaOH (w/v), 85 μL. After vortexing (10 s), NaCl (0.2 g) was added and an aliquot (1000 μL) of the resulting mixture was transferred to a 10 mL headspace vial, and extracted by solid-phase microextraction (SPME). The sample was pre-incubated at 60 °C for 2 min and then extracted using a polyacrylate fiber (85 μm), obtained from Supelco® (Bellefonte, USA), for 5 min at the same temperature at a mixing velocity of 500 rpm. The analytes were desorbed at 250 °C for 3 min. Gas-chromatographic separation was performed using a CP 3800 gas chromatograph (Varian, Middlesburg, The Netherlands) at an OV-1 column (30 m, 0.32 mm, 1 μm), from Ohio Valley (Marietta, USA). Carrier gas (helium) flow rate was 4 mL min⁻¹. The initial oven temperature was 70 °C, maintained for 3 min, and then increased 20 °C min⁻¹ until 100 °C, which was maintained for 6 min. The total running time was 10.5 min. Detection was made by a flame ionization detector (FID), kept at 250 °C. The retention times were 7.5 and 9.5 min for o-C and IS, respectively. The detection and quantification limits were 0.001 and 0.003 g of HA per g of creatinine; and 0.04 and 0.06 mg L⁻¹ for o-C, respectively.

2.5. Creatinine concentration

Creatinine concentration was measured by spectrophotometry, as previously described in the literature [31] with some modifications, using commercial laboratory kits (Doles reagents, Goiânia, GO, Brazil).

2.6. Cotinine levels

The urinary cotinine was analyzed according to Catteano et al. [32]. Briefly, 2 ml of urine sample, was alkalized with NaOH. After extraction with dichloromethane, the samples were dried and recovered with mobile phase and injected into the HPLC system.

2.7. Comet assay

A standard protocol was adopted for comet assay preparation and analysis [33,34]. Slides were prepared by mixing 5 μL whole blood and 95 μL low melting point agarose (0.75%). The mixture was poured on 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 (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, with freshly made 1% Triton X-100 and 10% DMSO) for a minimum of 1 h and a maximum of 5 days. Subsequently, the slides were incubated in freshly made alkaline solution (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min. The DNA was electrophoresed for 20 min at 25 V (0.9 V/cm) and 300 mA, and the solution was neutralized with 0.4 M Tris (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, consisting of whole blood and whole blood mixed with methyl methanesulfonate to 8 × 10⁻⁶ M final concentration, respectively. This mixture was incubated at 37 °C for 2 h. Images of 100 randomly selected cells (50 cells from each of the two replicate slides) were analyzed from each sample. Each electrophoresis run was considered valid only if the negative and positive controls yielded the expected results. The damages were visually scored according to tail size into five classes, ranging from no tail (0) to maximal (4) long tail, resulting in a single DNA damage score for each subject and, consequently, for each study group. Therefore, a group damage index (DI) could range from 0 (all cells with no tail, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4). All electrophoresis analyses performed in this study showed the following results for positive and negative controls: DNA DI for positive control, 380–400, and for negative control, 0–4.

2.8. Micronucleus assay

For the micronucleus assay in buccal cells, the heads of the brushes used to collect the samples were individually placed into separate tubes containing 20 mL buccal cell (BC) buffer (Tris–HCl, 0.01 M; EDTA tetra sodium salt, 0.1 M; NaCl, 20 mM)
at pH 7.0. Cells of both right and left cheeks were mixed and centrifuged at 1500 g for 10 min. The supernatant was removed and replaced with 10 mL fresh BC buffer. Cells were spun and washed more three times. One sample was applied to clean microscope slides and fixed with absolute methanol. The slices were stained with 5% Giemsa solution. The criterion of scoring cells with MN was the same as described in the literature [35]. We scored one thousand cells for each sample. Results were expressed as the frequency of abnormal cells per 1000 cells.

2.9. Lipid peroxidation

Quantification of lipid peroxidation was performed by measuring the MDA levels by HPLC with VIS detection, as described by our group [36]. This method analyzed the MDA levels with alkaline hydrolysis at 532 nm. The mobile phase in the HPLC was a mixture of Milli-Q water and methanol (50:50, v/v). The flow rate was maintained isocratically at 0.6 mL/min, the absorbance of the eluent was monitored at 532 nm and the total running time was 8 min. The column was thermostated at 40 °C in a thermostatization system for chromatographic columns (Chromar®). The results were expressed as μM.

2.10. Protein carbonyl levels

The protein carbonyl levels were determined according to previous study [37]. The method is based on the reaction of the carbonyl groups with 2,4-dinitrophenhydrazine (DNPH) to form 2,4-dinitrophenhydrazo. Brieﬂy, proteins were precipitated by the addition of 20% TCA, resolved in DNPH, and the absorbance read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl mg protein−1. Total protein concentrations were determined by Biuret method (L�astet Kit, Lagoa Santa, Minas Gerais, Brazil) using bovine serum albumin as standard.

2.11. IMA levels

Serum ischemia-modified albumin (IMA) was measured by the albumin cobalt binding test on a Cobas MIRA® Plus analyzer according to a previously described and validated method [38,39]. Patient plasma (95 μL) was pipetted into the reaction cuvette on the Cobas MIRA® Plus analyzer. An aliquot (5 μL) of 16.8 mM CoCl₂ solution was added to 20 mL of barbital buffer (pH 8.6) and was incubated for 25 s. The sample/cobalt/buffer mixture was incubated for 275 s to allow binding of cobalt to albumin, and then a blank reading optical measurement was made at 500 nm. An aliquot (25 μL) of 9.7 mM dithiothreitol (DTT) was added 25 s later. DTT reacts with unbound (non-N-terminal-sequestered) cobalt to form a colored product. The final reaction mixture was incubated for an additional 100 s and read at 500 nm. All incubations were performed at 37 °C. The total assay time once the sample was pipetted was 75 min. IMA results were expressed in absorbance units (AU).

2.12. Albumin levels

The serum albumin (ALB) levels were measured by a standard method with commercial laboratory kits (L�astet, Minas Gerais, Brazil).

2.13. Hepatic enzymes determination

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified by colorimetric methods, using commercial laboratory kits (Doles reagents, Goiânia, GO, Brazil).

2.14. Statistical analysis

Statistical analysis was performed using Statistica 6.0 software system (Statsoft Inc., 2001). The results were expressed as mean ± standard error mean (SEM). Comparisons between groups were achieved by the Mann–Whitney U-test. Correlation tests were performed according to Spearman’s rank following the variables distribution. Genetic damage related to exposure level was evaluated by multiple linear regression analyses after adjustment for age, smoking habits (based on cotinine levels in urine), alcohol consumption and exposure time. Values of p < 0.05 were considered significant.

3. Results

Painters enrolled in this study had a mean age of 28.9, ranging from 18 to 50 years old. In the control group the mean age was 29, ranging from 19 to 55 years old. The workers assessed were occupationally exposed to toluene in an average time of 46.15 ± 9.94 months. The characteristics of the studied groups, informed in the questionnaire, are presented in Table 1.

The biomarkers of toluene exposure are summarized in Table 2. Urinary hippuric acid levels were not statistically different between the groups (p > 0.05). Although HA concentrations were higher in painters, they were still below the biological exposure index (1.60 g g⁻¹ creatinine), according to ACGIH (American Conference of Governmental Industrial Hygienists). In terms of urinary o-C and blood toluene concentrations, all the values found in painters were below the allowed maximum levels established. The control group did not present measurable urinary o-C and blood toluene levels.

The comet assay data for painters and control group are presented in Table 3. Analyzing this data, it was possible to observe a significant increase in DNA damage index (DI) for painters in relation to control group (p < 0.001). The DI for six painters who were smokers did not reveal any significant difference when compared to non-smokers painters (n = 28) (p > 0.05). In the same way, in relation to alcohol consumption, the painters who were alcohol drinkers (n = 28) did not show a significant difference in DI when compared to non-alcohol drinkers (n = 6) (p > 0.05). Also, there was no significant difference in DNA damage index in smokers and alcohol consumers in the control group.

According to tail size score, the damages were classified into five classes. The results showed significant difference between the groups for all classes, except in class 4 (Fig. 1). In both groups, there was no significant difference between smokers and non-smokers or between alcohol drinkers and non-drinkers (data not shown).

In the MN assay, the frequency of abnormal cells did not show significant difference between painters and the control group (p > 0.05), being 2.74 ± 0.22 vs. 2.24 ± 0.29 MN/1000 cells, respectively.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of painters and controls.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confounding factor</td>
<td>Controls (n = 27)</td>
</tr>
<tr>
<td>Age (years) (mean ± SEM)</td>
<td>29.33 ± 10.56</td>
</tr>
<tr>
<td>Exposure time (months) (mean ± SEM)</td>
<td>–</td>
</tr>
<tr>
<td>Smoking habits (n [%])</td>
<td></td>
</tr>
<tr>
<td>Smokes</td>
<td>(3) (11.10)</td>
</tr>
<tr>
<td>No smokes</td>
<td>(24) (88.90)</td>
</tr>
<tr>
<td>Alcohol consumption (n [%])</td>
<td></td>
</tr>
<tr>
<td>Drinks</td>
<td>(21) (77.80)</td>
</tr>
<tr>
<td>No drinks</td>
<td>(6) (22.20)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Levels of biomarkers of exposure to toluene.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomarkers of exposure</td>
<td>Controls (n = 27)</td>
</tr>
<tr>
<td>Blood toluene (mg L⁻¹)</td>
<td>n.f.</td>
</tr>
<tr>
<td>Hippuric acid (g g⁻¹ creatinine)</td>
<td>0.41 ± 0.06</td>
</tr>
<tr>
<td>Ortho-creosol (mg L⁻¹)</td>
<td>n.f.</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SEM. BEI: biological exposure indices; n.f.: not found. * According to ACGIH, 2009.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Evaluation of DNA damage through Comet assay.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Damage index</td>
<td>Controls (n = 27)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 27)</td>
<td>39.4 ± 2.5</td>
</tr>
<tr>
<td>Smokers’ (n = 3)</td>
<td>49.0 ± 9.3</td>
</tr>
<tr>
<td>Non-smokers (n = 24)</td>
<td>38.2 ± 2.5</td>
</tr>
<tr>
<td>Alcohol drinkers (n = 21)</td>
<td>41.5 ± 2.7</td>
</tr>
<tr>
<td>Non-alcohol drinkers (n = 6)</td>
<td>32.2 ± 5.5</td>
</tr>
<tr>
<td>Painters (n = 34)</td>
<td>60.4 ± 3.6</td>
</tr>
<tr>
<td>Smokers (n = 6)</td>
<td>60.3 ± 10.0</td>
</tr>
<tr>
<td>Non-smokers (n = 28)</td>
<td>58.5 ± 3.8</td>
</tr>
<tr>
<td>Alcohol drinkers (n = 28)</td>
<td>60.1 ± 4.0</td>
</tr>
<tr>
<td>Non-alcohol drinkers (n = 6)</td>
<td>61.8 ± 9.2</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SEM. * (p < 0.001) compared with controls.
Fig. 1. DNA damage classes in the studied groups.

Table 4

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Controls (n = 27)</th>
<th>Painters (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (μM)</td>
<td>5.31 ± 0.22</td>
<td>10.02 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCO (nmol carbonyl mg protein&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.54 ± 0.02</td>
<td>0.71 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMA (ABSU)</td>
<td>0.48 ± 0.02</td>
<td>0.54 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Albumin (g dL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>4.28 ± 0.02</td>
<td>4.17 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SEM.
<sup>a</sup> (p < 0.001) compared with controls.
<sup>b</sup> (p < 0.05) compared with controls.

The results of lipid peroxidation analyses are shown in Table 4. MDA, the lipid peroxidation biomarker, was significantly higher in painters in comparison to controls, with MDA levels almost twice as high relative to the controls (p < 0.001). Regarding protein oxidation, PCO levels were increased in painters when compared to control subjects (p < 0.05) (Table 4). Protein damage was also evaluated by IMA and albumin levels. Painters showed higher IMA concentrations (p < 0.05) and decreased ALB levels (p < 0.001) than the control group (Table 4). No significant difference in biomarkers of lipid and proteic damage was observed in relation to smoking and alcohol consumption (data not shown).

Analyzing the hepatic enzymes, painters showed increased AST and ALT levels when compared to the control group, 62.20 ± 4.91 UI mL<sup>−1</sup> vs. 41.40 ± 9.44 UI mL<sup>−1</sup> (p < 0.001); and 41.30 ± 3.79 UI mL<sup>−1</sup> vs. 36.30 ± 6.35 UI mL<sup>−1</sup> (p < 0.05); respectively.

Univariate analyses by Spearman correlation showed that DNA DI was positively associated with biomarkers of toluene exposure (blood toluene ($r^2 = 0.43; p < 0.001$) and urinary HA ($r^2 = 0.43; p < 0.001$)) (Fig. 2). Also, positive correlations were observed between DNA DI and MDA levels ($r^2 = 0.47; p < 0.001$), time of Fig. 2. Univariate correlations of DNA damage index with biomarkers of exposure to toluene: (a) blood toluene; (b) urinary hippuric acid. In both analyses: n = 61; $r^2 = 0.43; p < 0.001$.
Table 5
Variable associated with DNA damage index (regression model).

<table>
<thead>
<tr>
<th>Variable</th>
<th>β estimated</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood toluene (mg L⁻¹)</td>
<td>139.5</td>
<td>0.08</td>
</tr>
<tr>
<td>HA (g g⁻¹ creatinine)</td>
<td>6.08</td>
<td>0.25</td>
</tr>
<tr>
<td>MDA (μM)</td>
<td>-1.32</td>
<td>0.44</td>
</tr>
<tr>
<td>Albumin (g dl⁻¹)</td>
<td>-19.28</td>
<td>0.31</td>
</tr>
<tr>
<td>IMA (ABU)</td>
<td>-14.18</td>
<td>0.58</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.02</td>
<td>0.93</td>
</tr>
<tr>
<td>Time of exposure (months)</td>
<td>0.13</td>
<td>0.24</td>
</tr>
<tr>
<td>Cotinine (ng ml⁻¹)</td>
<td>0.01</td>
<td>0.21</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>-2.79</td>
<td>0.41</td>
</tr>
</tbody>
</table>

* Categorical variable.

* Continuous variable.

exposure ($r^2 = 0.55; p < 0.001$) and age ($r^2 = 0.27; p < 0.05$); moreover, DNA DI was also negatively associated with albumin levels ($r^2 = -0.36; p < 0.001$) (Fig. 3). Negative correlations were also observed between albumin levels and biomarkers of toluene exposure: blood toluene ($r^2 = -0.39; p < 0.001$) and urinary hippuric acid ($r^2 = -0.30; p < 0.05$). No correlation was observed between albumin and IMA levels ($r^2 = -0.02; p > 0.05$). When the correlations were performed within each group, no significant association was observed.

Table 5 shows the best-fit multivariate regression model evaluated to explain the increase in DNA DI in this study. This model of regression included as categorical variable: the biomarkers of toluene exposure (blood toluene and urinary hippuric acid), lipid peroxidation biomarker (MDA), ischemia modified albumin (IMA), albumin, age, exposure time, cotinine (smoking); and alcohol consumption as continuous variable. The model accounted 29.1% of DNA DI, with blood toluene levels presenting a tendency to explain the genetic damage index ($β$ estimate = 139.5; $p = 0.08$). The remaining variables evaluated in the model did not show significant values. Multiple regression models were not observed within each group of the present study.

4. Discussion

Genotoxic agents can cause a variety of types of DNA damage, including base modification, DNA adduction, single-strand breaks, double-strand breaks, intra- or inter-strand cross-links [40], which contribute to cancer development [16,23].

Although toluene is defined as a class 3 substance (not classifiable as carcinogenic to humans) [8], some studies have shown evidence for toluene genotoxic effects in exposed subjects [1,14,15]. Biological monitoring, using different exposure, effect, or susceptibility biomarkers, has a fundamental role in occupational risk assessment [41]. Several biomarkers are available to assess internal exposure to toluene; however, with the reduction of toluene content in some preparations coupled with better industrial hygiene conditions, more specific markers are required for biological monitoring of low-level exposure to toluene [42]. In addition to biomarkers of exposure, biomarkers of the effects caused by toluene, such as genotoxicity biomarkers, are needed to assess exposures to mutagens or genotoxic carcinogens [41].

In our study, painters and controls showed no significant difference in relation to HA levels, indicating the low specificity of this biomarker as a monitor to toluene exposure. Concerns about the value of hippuric acid as an exposure biomarker were raised in recent years, since occupational exposures to toluene have been gradually decreasing, and also because hippuric acid can be found in the urine of non-exposed subjects. The value of hippuric acid as a marker of occupational toluene exposure is further challenged by the presence of benzoate in some soft drinks added as a preservative, because benzoate is metabolized and excreted as hippuric acid in the urine [2,43].

![Fig. 3](image-url) Correlations between DNA damage index and: (a) MDA concentration; ($r^2 = 0.47; p < 0.001$); (b) exposure time ($r^2 = 0.55; p < 0.001$); (c) age ($r^2 = 0.27; p < 0.05$); (d) albumin levels ($r^2 = 0.36; p < 0.001$). In each analysis, $n = 61$. 
The values (HA and c-C) found in the urine of toluene-exposed painters were below the biological exposure index, and the same was observed for blood toluene. This fact could be explained by the low percentage of toluene present in paints. Even though toluene is the major organic substance found in paints, according to the components list presented by the industry, it represents only 15% of the composition of paints. With low levels of toluene in paints, less of this xenobiotic is absorbed and metabolized; this contributes to low levels of blood toluene and its urinary metabolites. Occupational exposure to toluene in this industrial unit might be considered very low, as confirmed by low levels of toluene exposure biomarkers, much lower than the respective biological exposure index.

The comet assay, a sensitive method of measuring DNA damage [33] showed that painters presented significantly higher DNA damage indices in comparison to the control group. These findings are consistent with previous studies [2,14,15], which also showed cytogenic damage under low level exposure conditions.

With regard to MN frequency in buccal cells, no significant difference was observed between painters and control subjects. This can be explained by the low levels of toluene found in paints and by the short-term (less than 4 year) exposures of the painters, which were insufficient to induce detectable damage. A recent report found a significant association between the time spent at work and MN frequency in buccal cell [44], thus highlighting the influence of exposure time on DNA damage.

According to Heuser et al. [15], differences in the findings of the comet and MN assays could be associated with the kinds of exposure and/or damage caused. Our results showed that genotoxic effects of toluene exposure could be detected by the comet assay, but did not affect MN frequency. The micronucleus assay is used for assessing DNA damage at the chromosomal level, and differs from the comet assay, which can detect repairable damage [45].

Despite low levels of toluene exposure, painters showed elevated oxidative damage. Our previous study has shown that workers exposed to paints also showed changes in lipid peroxidation and endogenous antioxidants [46].

Elevated MDA levels observed in painters could be linked to toxic effects of toluene by the formation of free radicals and reactive oxygen species (ROS) during its biotransformation, causing damage to biological membranes [47]. The ROS production during toluene biotransformation could also be responsible for protein oxidation. Our results showed increased PCO levels in painters, indicating oxidative damage in proteins, reflecting the cellular damage induced by multiple forms of ROS [48,49]. In addition to the formation of protein carbonyls, another proteins oxidation was observed, with albumin as the main target. Elevated production of ROS during toluene biotransformation could transiently modify the N-terminal region of albumin and result in increased IMA levels [50,51].

Albumin, the most abundant serum protein, is a powerful extra-cellular antioxidant that contains —SH groups and works as a scavenger of reactive oxygen species [52]. Although the decreased levels of albumin found in painters could be due to oxidative stress, no correlation between IMA and albumin levels was observed. Accordingly, the low levels of albumin observed in painters group could be connected with low replacement of albumin by the liver, since occupational exposure can lead to hepatotoxicity [53]. In this study, the hepatic damage caused by toluene was evidenced by increased serum liver enzymes in the group of painters, which is accompanied by diminishing serum albumin levels.

There are many sources of DNA damage, but damage caused by free radicals and ROS may often be significant [54]. The linear correlation found between DNA DI and the biomarker of lipid peroxidation (MDA) is consistent with genotoxic effects related to oxidative stress. Furthermore, linear correlations were also observed between DNA DI and biomarkers of toluene exposure; higher levels of blood toluene and urinary HA were observed in subjects with higher DNA DI. Formation of ROS during toluene biotransformation could be involved in the genotoxic effects. The oxidative alterations present in lipids and protein give a measurement of exposure-induced oxidative stress that results in inflammation and damage to macromolecules, including DNA, proteins and lipids [55,56]. Although the comet assay is not able to discriminate the etiology of DNA damage, the genotoxic effects observed in painters may be due to production of free radicals during toluene biotransformation. The increase in DNA DI was also associated with increased age and exposure time.

According to multiple linear regression analysis, only blood toluene presented a tendency (p value near 0.05) to explain the increase in genetic damage. This result suggests that toluene is involved in the mechanism of reactive species formation, which could also be associated with genotoxic effects.

In conclusion, the results presented in this study showed that genetic damage can be associated with low levels of toluene exposure, being related with oxidative damage, age and time of exposure. Blood toluene seems to be the variable that best explains the increased DNA DI, but a larger group of workers should be evaluated, to test this hypothesis.

Conflict of interest

There is no conflict of interest.

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