Occupational risk assessment of paint industry workers

Abstract

Background: Thousands of chemical compounds are used in paint products, like pigments, extenders, binders, additives, and solvents (toluene, xylene, ketones, alcohols, esters, and glycol ethers). Paint manufacture workers are potentially exposed to the chemicals present in paint products although the patterns and levels of exposure to individual agents may differ from those of painters. The aim of the present study was to evaluate genome damage induced in peripheral blood lymphocytes and oral mucosa cells of paint industry workers. Materials and Methods: Genotoxicity was evaluated using the alkaline Comet assay in blood lymphocytes and oral mucosa cells, and the Micronucleus test in oral mucosa cells. For the micronucleus test in exfoliated buccal cells, no significant difference was detected between the control and paint industry workers. Results: The Comet assay in epithelia buccal cells showed that the damage index (DI) and damage frequency (DF) observed in the exposed group were significantly higher relative to the control group (P≤0.05). In the same way, the Comet assay data in peripheral blood leukocytes showed that both analysis parameters (DI and DF) were significantly greater than that for the control group (P≤0.05). Conclusions: Chronic occupational exposure to paints may lead to a slightly increased risk of genetic damage among paint industry workers.

Key words: Comet assay, micronucleus test, oral mucosa cells, paint industry workers, peripheral blood lymphocytes

INTRODUCTION

According to recent studies, occupational exposure to paint may cause an increased risk of several kinds of cancer, including lung, bladder and pancreas cancer, and lymphatic and hematopoietic tumors. These findings are consistent with the 1989 report issued by the International Agency for Research on Cancer, which classified painting as an occupationally related cause of cancer and provided further evidence that the risk of certain cancers is increased by exposures in the paint manufacturing process. However, occupational exposure in paint manufacture is not classifiable as to its carcinogenicity.
early 1970s. In the present study, we used the MN test in exfoliated mucosa cells and the single cell gel electrophoresis (SCGE) or the Comet assay because of the advantages these systems afford in the screening of DNA damage caused by environmental mutagens.

MN are acentric chromosome fragments or whole chromosomes delayed during mitotic cellular division, and appear in the cytoplasm of interphase cells as small additional nuclei. The MN test is faster and easier than metaphase analysis and it can be used both in vivo and in vitro in a variety of cells. This assay has also been shown to be a reliable and sensitive biomarker for human biomonitoring. The frequency of MN in human exfoliated cells is considered a useful biomarker of genotoxic effects in populations exposed to genotoxicants, through direct contact with ingested or inhaled compounds.

Briefly, in the Comet assay, which is a simple and sensitive method for studying DNA damage and repair, cells are embedded in agarose on a microscope slide, lysed with detergent and high salt to form the nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Then, they are electrophoresed in alkaline medium. In cells with increased DNA damage, the results are structures resembling comets, because the DNA migrates from the nucleiod, an event observable by microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks. The likely basis for this is that loops containing a break lose their supercoiling and become free to extend towards the anode. Similarly to other genotoxicity tests, the Comet assay is not predictive of individual cancer risk but represents a useful tool to evaluate early and still repairable genotoxic effects due to occupational or environmental exposure.

Considering that, to our knowledge, there is no published cytogenetic data concern paint industry workers in Brazil. Therefore, the objective of this study was to evaluate the genotoxic risk of these workers using the Comet assay in peripheral blood leukocytes and oral mucosa cells and the MN test in oral mucosa cells.

**MATERIAL AND METHODS**

**Study population and sample collected**

This study was approved by Brazilian National Ethical Committee on Research (Comissão Nacional de Ética em Pesquisa – CONEP, Protocols 061/2008) and informed written consent was obtained from each individual prior to the start of the study.

The study included 58 male workers of a paint manufacturing company employed in the sectors where they were occupationally exposed to solutions containing organic mixtures. The control group consisted of 30 healthy males with no occupational exposure that worked at the administrative function of the same company.

All the individuals examined in the study were required to answer a Portuguese version of a questionnaire from the International Commission for Protection against Environmental Mutagens and Carcinogens and participate in a face-to-face questionnaire which included standard demographic data (age, gender,) as well as questions relating to medical issues (exposure to X-rays, vaccinations, medications), life style (smoking, coffee, alcohol, diet,) and their occupation (number of hours worked per day, time exposed to organic solvents, use of protective measures). Individuals were selected for the two groups (control and paint exposed) in such a manner so as to ensure that except for occupational exposure to organic solvents, there were no marked differences between the members of the groups. Individuals who smoked more than five cigarettes per day for at least 1 year were considered smokers. The characteristics of the two groups are presented in Table 1.

Blood and urine samples were obtained from individuals in the two groups on the same day at the end of a normal shift during the workers’ periodical medical examinations by the nurses from a laboratory (BioLabor, Criciúma, Santa Catarina, Brazil). All blood samples were collected using venipuncture and heparinized vacutainers and processed as quickly as possible to avoid the damage associated with storage. The blood cell samples were transported to the university laboratory at or below 8°C and processed within 5 h of collection.

**Hippuric acid analysis**

As a biomarker of toluene exposure, 50 mL of urine samples were collected at the end of the working day and analyzed for hippuric acid (HA) using high performance liquid chromatography (HPLC) with UV-VIS detector in a commercial laboratory (Alvaro Laboratory, Cascavel-PR, Brazil).

**Analysis of Hematological parameters**

The following hematological markers were measured: leukocytes (granulocytes, lymphocytes, and monocytes), erythrocytes, hemoglobin, hematocrit.

All blood tests were analyzed in a laboratory (BioLabor, Criciúma, Santa Catarina, Brazil) according to standard hematological methods.

| Table 1: Demographic characteristics of the groups of study |
|-----------------------------------------------------------|
| Control group | Exposed group |
| Number of subjects | 30 | 58 |
| Age (Mean ± SD) | 28.24 ± 10.99 | 29.93 ± 9.98 |
| Smokers/non-smokers | 0/30 | 3/58 |
| Cigarettes/day | 0 | 10.33 ± 4.04 |
| Time of exposure (Mean ± SD) | - | 3.68 ± 3.29 |

Indian Journal of Occupational and Environmental Medicine - August 2011 - Volume 15 - Issue 2
**Genotoxicity tests**

**Comet assay in peripheral blood leukocytes**

The alkaline Comet assay was performed as described by Singh et al., with the modifications suggested by Tice et al. Samples of 5 µL of whole peripheral blood were embedded in 95 µL of 0.75% low-melting point agarose and added to a microscope slide (two slides per donor) precoated with normal agarose (1.5% buffer solution). When the agarose solidified the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 4.0–4.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethylsulfoxide (DMSO) for a minimum of 1 h and a maximum of 2 weeks. After treatment with lysis buffer, to allow DNA unwinding, slides were incubated in a freshly made alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA; pH > 13) for 20 min in a horizontal electrophoresis tank and the DNA was electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA. Every step was carried out under indirect yellow light. After electrophoresis, slides were washed three times in a neutralization buffer (0.4 M Tris, pH 7.5) for 5 min, rinsed three times in distilled water, and left to dry overnight at room temperature. Slides were stained with silver nitrate as previously described by Villala et al.: the slides were fixed for 10 min in trichloroacetic acid 15% w/v; zinc sulfate 5% w/v, glycerol 5% v/v, rinsed three times in distilled water, and dried for 2 h at 37°C. The dry slides were re-hydrated for 5 min in distilled water, and then stained (sodium carbonate 5% w/v, ammonium nitrate 0.1% w/v, silver nitrate 0.1% w/v, tungstosilicic acid 0.25%, formaldehyde 0.15% w/v, freshly prepared in the dark), and constantly shaken for 35 min. The stained slides were rinsed twice with distilled water, and submerged in the stop solution (acetic acid 1%), rinsed again, and immediately coded for analysis in an optical microscope. Images of 100 randomly selected cells were analyzed per individual. Cells were scored visually into five classes, according to tail size and shape (from undamaged – 0, to maximally damaged – 4), and a value (damage index (DI)) was assigned to each Comet according to its class. DI thus ranged from 0 (completely undamaged: 100 cells×0) to 400 (with maximum damage: 100 cells×4). The damage frequency (DF) (%) was calculated based on the percentage of damaged cells (0–100%). International guidelines and recommendations for the Comet assay consider that visual scoring of comets is a well-validated evaluation method. It has a high correlation with computer-based image analysis. Negative controls were processed together with workers’ samples and analyzed by one investigator.

**Comet assay in epithelial buccal cells**

Exfoliated buccal mucosa cells were collected by swabbing the right inner cheek of the individuals with a moistened wooden tongue depressor and was smeared over clean slides containing two drops of physiological solution. Cells were fixed in a methanol–acetic acid (3:1) solution for 10 min, dried at 50°C in a chamber for 5 min, and stained with Giemsa 5% (phosphate buffer solution, pH 5.8). Then the slides were washed in distilled water and stained with Fast green for 1 min, washed again, and stained with total Giemsa for 1 min. After this, they were washed in distilled water again and dried at room temperature.

The criteria used for MN analysis were those of Tolbert et al. and Titenko-Holland et al., i.e. for a structure to be considered as a micronucleus it must be: (a) less than one third of the diameter of the main nucleus; (b) be in the same plane of focus as the main nucleus e; (c) have the same color, texture, and refraction as the main nucleus; (d) have a smooth oval or round shape; and (e) be clearly separated from the main nucleus. Only cells that were not smeared, clumped or overlapped, and those who contained intact nuclei were included in the analysis. According to Tolbert et al. and Gomez-Arroyo et al., exfoliated buccal cells undergo degenerative processes which can produce anomalies that are difficult to distinguish from MN (binucleates, pycnosis, karyorrhexis, and karyolysis). In our study, these were excluded from the micronucleus analysis and all the slides were coded to blind analysis. The MN frequency was estimated based on the number of normal exfoliated buccal cells counted using a bright-field Zeiss microscope at a magnification of 1000×. For each volunteer, 2000 buccal cells (i.e. 1000 from each of the duplicate slides) were scored.

**Statistical analysis**

The normality of variables was evaluated by the Kolmogorov–Smirnov test; χ² and t-tests were used to compare the demographic characteristics of study populations. The statistical analysis of differences in HA, MN test, and DNA damage measured by the Comet assay were carried out using the non-parametric Mann–Whitney U-test. Correlations between different variables were determined by the Spearman rank correlation test, when appropriate. The critical level for rejection of the null hypothesis was considered to be a two-tailed P value of 5%. All analyses were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com.

**RESULTS**

The main characteristics of the two groups studied are presented in Table 1. No significant differences were observed between the mean age of subjects in the different groups (Student’s t-test). Regarding smoking habits, in the exposed
group just three subjects smoking an average of $10.33 \pm 4.04$ cigarettes per day, while in the control group all individuals were non-smokers. The duration of exposure in the exposed group was $3.68 \pm 3.29$ years, ranging from 0.5 to 12 years. Analysis of questionnaires revealed that all paint industry workers used silicone gloves to prevent skin contact with organic solvents, glasses, and breathing masks. We also observed that the work areas in the company were equipped with ventilation devices.

In relation to hematological parameters, Table 2 reports that no significant differences were found between the two groups, and both presented normal hematological values similar to reference values observed in the literature for several other Brazilian populations as described by.[21]

The comparison of the mean values (g/g creatinine) of urine HA level of the control and exposed group is shown in Figure 1. A significant increase in HA levels was observed in the exposed group relative to the controls ($P \leq 0.05$, Mann–Whitney U-test).

Table 3 shows the data obtained using the three cytogenetic assays for control and exposed groups, and that were analyzed using the Mann-Whitney U-test. For the micronucleus test in buccal exfoliated cells, no significant difference was detected between the control and paint industry workers. The Comet assay in epithelial buccal cells showed that the DI and DF observed in the exposed group were significantly higher relative to the control group ($P \leq 0.05$). In the same way, the Comet assay data in peripheral blood leukocytes showed that both analysis parameters of this assay (DI and DF) were significantly greater than that for the control group ($P \leq 0.05$). In addition, no significant differences were observed between smokers and non-smokers in the exposed group for all cytogenetic analysis. Also, no significant differences were observed in relation to exposure time for all assays [Table 4].

**DISCUSSION**

Many approaches and techniques have been developed for monitoring human populations exposed to environmental mutagens. The traditional approach has been to use the readily available blood cells (e.g., lymphocytes and red blood cells) as biomarkers to document mutagenic effects.[11] Although long-term diseases are not expected from the affected blood

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**Table 2: Hematological parameters in study groups (Mean ± SD)**

|                      | Control group (n=30) | Exposed group (n=58) | $P^*$   |
|----------------------|----------------------|----------------------|---------|
| Leukocyte count (x 10^6/µl) | 6.90 ± 1.27          | 7.41 ± 1.54          | 0.1380  |
| Granulocytes (%)     | 55.28 ± 9.42         | 51.07 ± 7.87         | 0.0894  |
| Lymphocytes (%)      | 44.53 ± 7.92         | 39.86 ± 8.90         | 0.0592  |
| Monocytes (%)        | 3.40 ± 1.77          | 3.88 ± 2.20          | 0.3860  |
| Erythrocytes count (x 10^12/µl) | 4.99 ± 0.22       | 4.93 ± 0.38          | 0.4551  |
| Hematocrit (%)       | 47.13 ± 2.59         | 45.60 ± 3.20         | 0.0630  |
| Hemoglobin (g/dl)    | 15.66 ± 0.16         | 15.24 ± 0.22         | 0.1335  |

*$\text{Student's t-test}$

**Table 3: Mean values (± SD) obtained by MN test and Comet assay in control and exposed group**

|                      | MN in 2000 epithelial buccal cells | Comet assay in 100 epithelial buccal cells | Comet assay in 100 leukocytes |
|----------------------|-----------------------------------|------------------------------------------|-------------------------------|
|                      | Damage Frequency | Damage index | Damage Frequency | Damage index | Damage Frequency | Damage index |
| Control group (n=30) | 3.88 ± 4.58      | 13.56 ± 12.69 | 18.81 ± 18.93   | 2.71 ± 2.27 | 2.64 ± 2.13   |
| Exposed group (n=58) | 6.63 ± 8.13      | 22.38 ± 17.28 | 33.43 ± 30.18*  | 6.33 ± 5.31** | 6.90 ± 6.11*  |

*Data significant relative to control group at $P \leq 0.05$, and **$P \leq 0.01$ (Mann-Whitney U-test, two-tail).

**Table 4: Mean values (± SD) obtained by MN test and Comet assay in the exposed group according to smoking habit and time of exposure**

|                      | MN in 2000 epithelial buccal cells | Comet assay in 100 epithelial buccal cells | Comet assay in 100 leukocytes |
|----------------------|-----------------------------------|------------------------------------------|-------------------------------|
|                      | Damage Frequency | Damage index | Damage Frequency | Damage index | Damage Frequency | Damage index |
| All exposed group (n=58) | 6.63 ± 8.13      | 22.38 ± 17.28 | 33.43 ± 30.18   | 6.33 ± 5.31 | 6.90 ± 6.11 |
| Non-smokers (n=55)    | 6.63 ± 8.13      | 22.58 ± 17.87 | 34.84 ± 30.83   | 6.55 ± 5.35 | 7.15 ± 6.17 |
| Smokers (n=3)         | 6.67 ± 8.33      | 19.33 ± 16.17 | 26.00 ± 23.39   | 2.33 ± 2.08 | 2.33 ± 2.08 |
| $\leq 3.7$ years of exposure (n=36) | 7.57 ± 8.93      | 26.30 ± 18.81 | 34.56 ± 28.45   | 7.03 ± 5.40 | 7.75 ± 6.48 |
| $> 3.7$ years of exposure (n=22) | 5.14 ± 6.30      | 20.32 ± 18.12 | 31.59 ± 33.43   | 5.18 ± 5.08 | 5.50 ± 5.30 |
cells, it is generally accepted that the blood cells can be used as sentinel cells types to provide early warning signals for adverse health outcome. It is also suitable to determine whether the biomarker effects observed in blood cells are consistent with those in available target cells. In this paper, we provided a study using two types of cells and two genotoxicity assays to evaluate the occupational exposure of paint industry workers.

Paint industry workers are exposed to complex mixtures of organic solvents, heavy metals such as lead, zinc, chromium, cadmium, and many other compounds with potential mutagenic properties, such as phthalic acid and chlorophenols.[9] Because the main solvent present in the mixtures of organic solvents used in the paint production process is toluene, we analyze the HA concentration in urine of the paint industry workers. Our data indicate a higher mean concentration of this toluene metabolite appearing in the urine of these workers in relation to the control group, and these levels confirmed exposure to toluene among paint industry workers [Figure 1]. However, the HA values observed for all volunteers can be considered low according to NR-7[22] (up to 1.5 g/g creatinine). Nevertheless, similar result was found by Pelcova et al.,[23] in printers exposed to toluene in Poland. The HA in urine in this group of printers were significantly higher than in controls; however, the levels nonetheless remained lower than the Czech limit for occupational settings (i.e., 2.5 g/L of urine). In addition, De Rosa et al.[24] carried out a monitoring of subjects working in a printing company, who were exposed to toluene, using urine samples collected before and after the work shift for the determination of HA. They found many correlations between levels of HA in urine and the environmental samples of toluene collected at the company, concluding that HA is a valid test for evaluating even low exposures to toluene.

Some of the biological effects of exposure to organic solvents are hematological changes.[25] These effects may result in a decreased production of red blood cells, white blood cells, and platelets.[26] However, in contrast to the results of Beving et al.,[27] with painters in Sweden, the values obtained in our study for hematological parameters showed no significant differences between control and exposed group.

The influences of age, sex, and smoking on DNA damage are well-known problems in industrial monitoring.[9,28] However, in this study these factors could be excluded, in both groups, the mean age was similar, all persons were men, and just three workers had smoked habits although in this case the values obtained for cytogenetic tests were not different from control subjects. Similarly, Silva et al.[29] concluded that smoking habits do not represent a significant factor in terms of production of the various types of chromosome aberrations found in their occupational monitoring with car painters. The results obtained in this study show that there is no exposure-related induction of MN in buccal epithelia cells of workers exposed to solvents in the paint manufacturing industry. Although only a few studies have been conducted with paint workers, data reported using these cells indicate positive results.[6,26,30] The use of the MN test in exfoliated cells has substantially increased, since it is considered a useful biomarker of genotoxic effects in population exposed to genotoxicants through direct contact with ingested or inhaled compounds. It must be recalled that epithelial cells are highly proliferative and are the origin of more than 90% of cancers, for which their use in biomonitoring can be really useful.[11] On the other hand, the Comet assay values for the paint industry workers were significantly higher than the values of the control group both in the peripheral blood and buccal exfoliated cells. Positive results in the Comet assay do not always correspond to positive results in the MN test, especially when the exposure to genotoxic agents is small. The Comet assay usually detects more defects than the MN test.[11] The positive results in the Comet assay and MN tests are due to different mechanisms; the MN test detects injuries that survive at least one mitotic cycle, while the Comet assay identifies repairable injuries or alkali-label sites.[31,32] Consequently, Goethem et al.[11] suggested the use of both the MN test and the Comet assay.

The possibility of cytogenetic damage in various occupations exposed to organic solvents has been discussed in several papers.[6,8,26,29,30,32-35] The increasing use and diversity of solvents raises concern about possible risks in occupational exposure. Several earlier reports suggest harmful effects in subjects occupationally exposed to paint and their components. Madhavi et al.[5] reported that occupational exposure to lead-based paints has been associated with an increase in the frequency of CA in the workers when compared to the controls. A monitoring study was designed by Pinto et al.[36] to determine occupational exposure risk in outdoor painters. Painters showed CA and SCE in lymphocytes and MN in oral epithelia cells greater than in the control group. Diaz et al.[36] analyzed lymphocytes and oral mucosa cell MN in 21 Cuban paint industry workers. Both MN assays showed the same results, i.e. a statistically significant difference between the workers and the control group. Another study reporting CA in lymphocytes from car painters in Brazil showed that there was a significantly higher frequency of aneuploidies and chromosome deletions in the peripheral lymphocytes of car painters than in control subjects.[29]

The MN test and the Comet assay were applied to exfoliated buccal cells in order to evaluate the genotoxic risk associated with occupational exposure of 10 car painters by Martino-Roth et al.[10] Highly significant effects of occupational exposure were found in this study with both the MN test and the Comet assay. On the other hand, Cárdenas-Bustamante et al.[35] investigated the degree of exposure to organic solvents.
and related genotoxic consequences in paint factory workers, determining MN frequency in lymphocytes and DNA damage using the Comet assay. They found no statistical differences regarding genetic biomarkers between exposed and non-exposed workers.

A major problem in interpreting biomonitoring studies is estimating the degree of exposure. Possible abuse or misuse could lead to significant levels of exposure. Until now, many biomonitoring studies have been performed in people from different regions and under a variety of exposure conditions, using several different biomarkers. In this context, it is not surprising that the results obtained by different authors have also shown variability. Also, since workers are frequently exposed to complex mixtures of organic solvents present in paints, it is difficult to attribute the genotoxic damage to any particular chemical or compound. Thus, the DNA damaged observed in our study and in all these listed above should not be attributed only to one compound, but to the cumulative effect of many chemical compounds that are used in paint products.

In the present study, even though paint workers had said that they used adequate personal protection equipment, an increase in HA concentration in urine was observed together with an increase in Comet assay values, both for leukocytes and buccal cells. Organic solvent levels in the samples were apparently low, which is consistent with the absence of mutagenicity and the presence of only genotoxicity in cells. It can be concluded from this study that occupational exposure to paints may lead to a slightly increased risk of genetic damage among paint industry workers. Due to these considerations and the complex multi-composition of paints and solvents, hygienic measures were suggested. A better understanding of variables related to cytogenetic damage would greatly reduce the uncertainty in the carcinogenic risk assessment among paint industry workers.

ACKNOWLEDGMENTS

The authors express their gratitude to all the individuals who volunteered to participate in this study. We especially thank the chairman of the paint manufacturing company and also Marcelo Comin for his valuable help during the sampling.

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Cite this article as: de Oliveira HM, Dagostim GP, Mota A, Tavares P, da Rosa LA, de Andrade VM. Occupational risk assessment of paint industry workers. Indian J Occup Environ Med 2011;15:52-8.

Source of Support: Programa de Pós-Graduação em Ciências da Saúde - Universidade do Extremo Sul Catarinense (UNESC) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Conflict of Interest: None declared.
