ORIGINAL ARTICLE

Biomarkers of susceptibility and effect in car painters exposed to organic solvents

Biomarcadores de susceptibilidad y efecto en pintores de carros expuestos a solventes orgánicos

Marcela Varona-Uribe1, Milcíades Ibáñez-Pinilla1, Leonardo Briceno-Ayala1, Diego Herrera1, Lilian Chuaire-Noack2, María Martínez-Agüero3, Magda Carolina Sánchez Corredor5, Ruth Palma-Parra4, Diana Narvaez5 and Helena Groot de Restrepo5

marcela.varona@urosario.edu.co

1 Universidad del Rosario, Escuela de Medicina y Ciencias de la Salud, Bogotá, Colombia. 2. Universidad del Valle, Facultad de Salud, Escuela de Ciencias Básicas, Cali, Colombia. 3 Universidad del Rosario, Facultad de Ciencias Naturales y Matemáticas, Bogotá, Colombia. 4 Instituto Nacional de Salud, Bogotá, Colombia. 5 Universidad de los Andes, Laboratorio de Genética Humana, Bogotá, Colombia.

Abstract

Introduction:

Car painters are routinely exposed to organic solvents classified as carcinogenic and mutagenic substances.

Objective:

To characterize the population susceptibility and evaluate the genotoxic effects of exposure to organic solvents.

Methods:

A cross-sectional study comparing a group of car painters exposed to organic solvents with a non-exposed group. CYP2E1 polymorphisms and the presence of micronuclei in lymphocytes were determined.

Results:

One hundred twenty-two workers participated in the study: 62 who worked in car paint shops and were exposed to solvents, and 60 who were not exposed. There were statistically significant differences between the two groups regarding micronucleated cells and nucleoplasmic bridges frequencies (p = 0.042 and p = 0.046, respectively; exact likelihood ratio). Significant differences were found at the interaction between the CYP2E1 genotype c1c1 and occupational exposure to solvents, with higher frequencies of micronuclei (p = 0.013) and micronucleated cells (p = 0.015). However, when the frequencies of micronuclei, micronucleated cells and nucleoplasmic bridges in the exposure group were compared between the c1c1 and c2c2/c1c2 allele groups of the CYP2E1 polymorphism, statistically significant differences were found.

Conclusions:

This study confirms that when workers with CYP2E1 polymorphisms, specifically the c1c1 genotype, are exposed to organic solvents, they are more likely to have somatic cell mutations, a condition associated with increased susceptibility to diseases such as cancer.
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Resumen

Introducción:

Los pintores de vehículos automotores están rutinariamente expuestos a agentes como los solventes orgánicos, capaces de producir efectos mutágeneos y carcinógenos.

Objetivo:

Caracterizar la susceptibilidad poblacional y evaluar los efectos genotóxicos debidos a la exposición a solventes orgánicos.

Métodos:

Estudio de corte transversal que comparó a un grupo de pintores de carros expuestos a solventes orgánicos con un grupo de personas no expuestas. Fueron determinados tanto los polimorfismos de CYP2E1 como la presencia de micronúcleos en linfocitos.

Resultados:

Participaron 122 personas, 62 trabajadores de talleres de pintura de autos expuestos a solventes y 60 personas no expuestas. Con relación al cuestionario Q 16, 32% de los expuestos refirieron síntomas sugestivos de neurotoxicidad. Las frecuencias de células micronucleadas y de puentes nucleoplásmicos fueron significativamente mayores en los expuestos que en los no expuestos: \( p = 0.042 \) y \( p = 0.046 \), respectivamente (Razón de verosimilitud exacta). Fueron halladas diferencias significativas en la interacción de CYP2E1 (c1c1) y la exposición ocupacional a solventes, con mayores frecuencias de micronúcleos \( (p = 0.013) \) y de células micronucleadas \( (p = 0.015) \).

Conclusiones:

Este estudio reafirma que los trabajadores expuestos a solventes orgánicos con polimorfismos de CYP2E1, específicamente con genotipo c1c1, tienen mayor probabilidad de presentar mutaciones en las células somáticas, condición asociada con una mayor susceptibilidad a enfermedades como el cáncer.

Introduction

Workers in car paint shops are exposed to complex mixtures of organic solvents which include chemicals such as xylene, methanol, toluene, benzene, methylene chloride, acetone, and polycyclic aromatic hydrocarbons, among others.\(^1\). Once inside the body, organic solvents undergo detoxifying metabolic reactions, through the activity of enzymatic families such as the cytochrome P450 family. This process gives rise to toxic intermediates such as free radicals and reactive oxygen species, which can cause primary DNA lesions. When these lesions are not repaired, they can become fixed and give rise to mutations in somatic cells, which increases the risk of diseases such as cancer.\(^2\).

Therefore, organic solvents are considered as potent mutagens, clastogens, carcinogens and teratogens. In particular benzene exhibits hemotoxic, immunotoxic, cytoxic and genotoxic properties, and is associated with an increased risk of leukemia. This situation led the International Agency for Research on Cancer (IARC) to classify vehicle painting as a carcinogenic industrial process.\(^8\).
The effect of exposure to organic solvents can be evaluated using genotoxic effects and genetic susceptibility biomarkers. In this regard, the cytokinesis-block micronucleus assay (CBMN) is currently one of the most frequently used techniques to assess chromosomal breaks or losses resulting from exposure to genotoxic agents.\textsuperscript{9-11} which may be seen as micronuclei or small extranuclear bodies within the cell. Exposure to clastogenic agents increases the frequency of micronuclei; therefore, micronuclei are indicators of genotoxic and cytotoxic effects.\textsuperscript{11} since they assess the risk of cancer due to occupational exposure to solvents. The CBMN additionally allows to score other nuclear anomalies apart from micronuclei that reflect genomic instability such as nucleoplasmic bridges, a biomarker of DNA misrepair and/or telomere end-fusions and nuclear buds associated with expulsion of amplified DNA or unresolved DNA repair complexes.\textsuperscript{12} The CBMN has demonstrated that certain workers routinely exposed to hexane, toluene, and benzene (such as shoemakers, workers at oil refineries, and photocopiers)\textsuperscript{7,13-16} have genotoxic damage caused by exposure to these substances.\textsuperscript{3,17}

Furthermore, during the first phase of their metabolism, organic solvents induce oxidation reactions mediated by cytochrome P450, an enzyme encoded by a gene whose polymorphisms have been associated not only with altered transcriptional regulation and increased expression of certain genes, but also with an increased frequency of chromosomal aberrations and cancer susceptibility.\textsuperscript{18} One of the most studied genetic variation is the restriction fragment length polymorphism PstI/RsaI located in the 5\textsuperscript{prime} flanking region of the gene. The mutant allele (c2) has been associated with an altered enzyme expression of the enzyme.\textsuperscript{19} Hence, genetic characterization of the workers in car paint shops is important to describe the possible toxic effect of organic solvents exposure.

In Colombia, occupational exposure to solvents constitutes a public health problem. Most car paint shops belong to the informal sector of the economy, which explains why their workers do not observe biosecurity measures or have medical services focused on the prevention of health risks. In addition, the low level of schooling is reflected in their lack of knowledge about the potential protection measures and the consequences that exposure to organic solvents have on health. It is usual for these informal workers to make the paint on their own, ignoring national or international quality standards. Furthermore, their workday may extend beyond the eight hours a day established by Colombian law, conditions that also increase the risks of genotoxic exposure. Although some studies have been conducted worldwide on this topic, including Colombia,\textsuperscript{20,23} the relationship between the CYP2E1 polymorphisms and the presence of micronuclei in car paint shop workers have not been examined, which could be used as biomarkers of genotoxic damage and predictors of cancer risk. Consequently, it is necessary to characterize the health and work conditions of individuals occupationally exposed to organic solvents used in car paint workshops in Bogota, Colombia, to alert to the possible onset and extent of early genetic damage, as well as to identify the risk of long-term health problems and to take preventive actions aimed at promoting health. To this end, the behavior of genotoxicity and genetic susceptibility biomarkers was evaluated to suggest preventive measures to minimize exposure and thus the likelihood of presenting genotoxic damage.

**Materials and Methods**

**Study population**

This was a cross-sectional study with a sample population from Bogota, Colombia. The sample size was calculated according to the results of Heuser et al.'s 2007 study,\textsuperscript{4} which used the Damage Index (DI) variable as a measure of genetic damage, taking a comparison of means of two populations with an unknown variance and an estimated statistical power of 95%, considering a 20% of loss. The population sample of exposed individuals was collected in the Barrios Unidos locality, in car workshops with exposure to the organic solvents used in the production of car paint, while controls were exclusively obtained from an educational...
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Institution in the same neighborhood, without exposure to solvents. Based on the universe of the exposed population, previously established by the local councilors, visits were made to the owners and/or administrators of the painting workshops. Those workers who voluntarily agreed to participate were included in the study until completing the sample size of the exposed group. A similar procedure was used in the educational institution to recruit participants to the group of non-exposed individuals. The exposed group was consisted of 62 adult male workers occupationally exposed to solvents for at least five years, without relevant medical history (such as cancer, hepatitis B, recent or prolonged medical treatment, or prior exposure to radiation). Subjects who were regular smokers or drinkers and used any type of psychoactive drugs, and whose had an extra-occupational exposure were excluded. The non-exposed group consisted of 60 individuals recruited from the educational institution, which were selected under the same criteria except exposure to solvents. In both groups, workers consented to share their information on personal, health and work aspects. There was also verification that, in the workshops studied, the procedures involved in paint mixing and application had not been significantly altered in the last 20 years. A pilot study was conducted in 10% of the sample before starting data collection, to standardize timing and actions.

Data collection

A questionnaire was filled out for each worker, which included variables such as age, occupation, date of employment, smoking and alcohol habits, frequency and time of exposure to organic solvents and medical history. The Q 16 questionnaire was included to evaluate neurotoxic symptoms and, with more than six affirmative answers, determines the suspected cases. This questionnaire was applied directly by the researchers to each of the participants.

The cytokinesis-block micronucleus assay

Genotoxicity as a measure of chromosomal damage in human lymphocytes was determined by the Cytokinesis-block micronucleus assay (CBMN). Briefly, a blood sample of 5 mL per individual was collected on the last workday of the week. 500 μL of blood were added to RPMI 1640 culture medium, from which two cell cultures were established. After 44 hours, cytochalasin B was added to each culture to block cytokinesis. At 72 hours of culture, cells were treated for five minutes with a hypotonic KCl 0.075 M solution, and subsequently prefixed and fixed with solutions of methanol and acetic acid, (3:5) and (5:1), respectively, to finally obtain four slides of each culture which were then stained with Giemsa. The frequencies of micronuclei, micronucleated cells, nucleoplasmic bridges and nuclear buds were analyzed in 2000 binucleated cells for each of the samples.

Determination of CYP2E1 genotype

A polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) was used for determining genetic polymorphisms of CYP2E1 enzyme. DNA was amplified with primers 5’-CCAGTCGAGTCTACATTGTCA-3’ and 5’-TTCATTCTGTCTTCTAACTGG -3’, and a 410 bp fragment was obtained. To recognize the c1 and c2 alleles in the CYP2E1 genotype, the amplified DNA was digested with RsaI endonuclease at 37 °C for one hour. Fragmented DNA was separated on 2% agarose gel. The c1c1 homozygous genotype corresponds to a fragment of 360 and 50 bp. In the c2c2 homozygote there is a 410 bp fragment, and in the c1c2 heterozygote all the three fragments are present.

Statistical analysis

To minimize potential biases in sampling, the results of exposed workers and non-exposed individuals were paired by age group. The data obtained were analyzed with the Statistical Package for the Social Sciences (SPSS) program. A descriptive analysis was performed, determining frequency distributions and measures of central tendency and dispersion according to the measurement levels of the workers’ variables, and biological measurements.
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The normal distribution of the data was established using Kolmogorov-Smirnov with a significance level of 10% ($p=0.10$). Associations between occupational exposure to organic solvents and occupational variables, neurological symptoms and genetic biomarkers was evaluated by Pearson's chi-square asymptotic test of independence or the Fisher's exact test and likelihood ratio (expected values <5). The nonparametric Mann Whitney-Wilcoxon test was used. An ordinal regression model (generalized linear models, GLM) was used to evaluate whether $CYP2E1$ and occupational exposure to organic solvents, and the interaction between these exposures, predicted the four outcomes of interest (i.e., micronuclei, micronucleated cells, nucleoplasmic bridges and nuclear buds). Allele frequencies were calculated and tested if were consistent with Hardy-Weinberg Equilibrium (HWE) by asymptotic tests in STATA v.12.0 software. The maximum level of significance was 5% ($p<0.05$)

Ethical considerations

The study followed the guidelines of the Declaration of Helsinki and Colombian standards for human research. The protocol was approved by the technical and ethics committees at the Instituto Nacional de Salud and the Universidad del Rosario in Bogota, Colombia. All participants signed an informed consent prior to conducting the survey and taking biological samples.

Results

One hundred twenty-two male workers participated in the study; 62 worked in painting workshops exposed to solvents and 60 were not exposed. Exposed workers had an average age of 43.9 ±12.4 years and an average exposure time of 21 ±11 years; meanwhile, the average age for unexposed workers was 40.1 ±10.6 years. There were no statistically significant differences ($p=0.067$) for age in the two groups of workers. In addition, 39% (n= 24) of them were working as painters, 19% (n= 12) as mechanics, 16% (n= 10) as auto body repairers, 8% (n=5) as colorists, and the rest worked in administrative areas (Table 1). Significant differences were found for occupational exposure, hygiene and protection elements variables being higher for the exposed group.

| Variable                  | Exposed (n = 62) | Non-exposed (n = 60) | p      |
|---------------------------|-----------------|----------------------|--------|
| Age (years)               |                 |                      |        |
| <29                       | 9               | 9                    | 0.203* |
| 30-39                     | 11              | 19                   |        |
| 40-49                     | 23              | 20                   |        |
| 50-59                     | 13              | 11                   |        |
| >60                       | 6               | 1                    |        |
| Occupational exposure     |                 |                      |        |
| Thinner                   | 57              | 1                    | <0.001 |
| Varsol                    | 29              | 2                    | <0.001 |
| Gasoline                  | 33              | 5                    | <0.001 |
| Acetone                   | 7               | 1                    | 0.062* |
| Glue                      | 31              | 1                    | <0.001 |
| Paint                     | 51              | 0                    | <0.001 |
| Paint solvent             | 50              | 0                    | <0.001 |
| Degreaser                 | 43              | 1                    | <0.001 |
| Cleanser                  | 40              | 6                    | <0.001 |
| Hygiene and personal protection |             |                      |        |
| Use of work clothes       | 47              | 7                    | <0.001*|
| Use of gloves             | 38              | 5                    | <0.001 |
| Use of respirator         | 45              | 2                    | <0.001 |
| Spare time activities     |                 |                      |        |
| Mechanic                  | 6               | 2                    | 0.273* |
| Carpentry                 | 3               | 3                    | 1.000* |
| Paint                     | 13              | 5                    | 0.073* |
| Laundry                   | 1               | 1                    | 1.000* |
| Stained glass             | 1               | 0                    | 1.000* |
| Pottery                   | 4               | 0                    | 0.119* |

* Fisher's exact test
The personal skin protection elements reported by workers were uniforms in 75% (47), followed by street clothes in 22% (14). Regarding the use of gloves, 28% said they were made of nitrile and an equal percentage of rubber. The remaining workers did not wear gloves.

Of the 45 workers using a respirator, 8% (4) used it with a filter and 35% (16) with a double filter. As for the type of filter, in 17% (8) it was impermeable to organic vapors while for the remaining percentage it was a face mask, an implement which does not provide appropriate respiratory protection.

Regarding the smoking variable, 31% (19) of the exposed and 48% (29) of the non-exposed had never smoked, while at the time of sampling, 20% of the exposed (13) and the non-exposed (12) smoked. No statistically significant differences between the two groups (*p*= 0.102) were found.

The presence of clinical manifestations was examined between the exposed and the non-exposed group (Table 2). The symptoms included in the Q 16 questionnaire were significantly higher in the exposed group when compared to the non-exposed group in 14 out of the 16 symptoms. The number of symptoms varied from 0 to 13, the median being significantly higher in the exposed than in the non-exposed group (4 vs. 0, *p* <0.001, Mann Whitney-Wilcoxon one-tailed exact test). The percentage with more than six symptoms was significantly higher in the exposed than in the non-exposed (17.7% vs. 3.3%, *p* = 0.009).

In addition, 6% (4) reported having been poisoned at least once with solvents. From the results of the Q 16 questionnaire, 32% (20) of the exposed and 5% (3) of the non-exposed presented more than six symptoms.

The CYP2E1 allele frequencies were in Hardy-Weinberg equilibrium (*p*=0.331). There were no statistically significant differences in the CYP2E1 polymorphism genotype characterization of the two groups (*p*=0.749, exact likelihood ratio test). Only one blood sample from the non-exposed group could not be processed (Table 3).

When compared the characteristics evaluated in the CBMN, there were statistically significant differences between the two groups regarding micronucleated cells and nucleoplasmic bridges frequencies (exact likelihood ratio *p*-values were 0.042 and 0.046, respectively) being higher for the exposed group. For the micronuclei and nuclear buds frequencies, there were no significant differences (exact likelihood ratio *p*-values were 0.296 and 0.933, respectively) (Table 4).
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Heterozygous individuals (c1c2) were grouped with homozygotes (c2c2) in the determination of the effect of each CYP2E1 genotype on chromosome damage, due to the low number of individuals with a homozygous genotype. Statistically significant differences were found for the interaction of c1c1 genotype and the occupational exposure to organic solvents, with higher frequencies of micronuclei and micronucleated cells (Table 5). No significant association was found for nucleoplasmic bridges and nuclear buds frequencies.

### Discussion

Exposure to organic solvents can cause serious health problems including poisoning or carcinogenesis, especially when benzene is involved. Biomonitoring of human populations occupationally exposed to those chemicals is essential to identify early biological effects and prevent the occurrence of associated chronic complications 26.

This study evaluated the genotoxic effects of exposure to organic solvents in a sample of workers routinely exposed to organic solvents due to work activities associated with car painting, and workers non-exposed to these agents. The average age observed in both groups were like those found in the available reports of the working population in Colombia, and in other studies with similar working populations 27. On the other hand, it is important to note that the time of exposure for the exposed group was approximately 21 years, and in addition, some of them performed tasks related to painting activities in their free time. These facts demonstrated that they were chronically exposed to chemicals, specifically aromatic hydrocarbons, which can cause long-term health effects, as has been reported in previous studies 28.
In the use of personal protection, presumably both employers and workers lacked knowledge regarding the harmful effects of these agents, since the exposed group clearly did not use basic protection, such as work clothing, gloves, and a respirator. This situation presents the added difficulty that only 17% wore nitrile gloves, which is the material recommended in the safety data sheets of these products. With respect to respiratory protection, the picture was no different, because only 32% used a filter respirator and, even more alarming, only 12% used a filter suitable for organic vapors. The above conditions are concerning, especially since they have been consistently described in other populations also exposed to organic solvents.

Manifestations such as emotional and mood disorders, as well as working and short-term memory disorders, have been associated with chronic exposure to solvents. Statistically significant differences between the exposed and non-exposed groups were found related to some symptoms included in the Q16 questionnaire such as being forgetful, feeling angry for no reason, feeling pressure in the chest, having trouble concentrating, feeling sad or depressed without reason, among others. In addition, a third of those exposed (32%) reported more than six of the symptoms included in that questionnaire, which would suggest a risk of neurotoxicity.

With respect to genotoxicity evaluated through the CBMN, significant differences were also observed between exposed and non-exposed, specifically in the micronucleated cells and nucleoplasmic bridges frequencies, indicating the high degree of genotoxicity that can be attributed to the organic solvents used in the preparation of car paint. It is worth mentioning that the CBMN expresses cumulative DNA damage associated with chronic exposures such as those presented by individuals in the exposed group. This damage accumulates within the lymphocytes for long periods, turning them into sentinels that allow the identification of genotoxicity, especially through assays like this one, that has become one of the standard cytogenetic tests in the field of genetic toxicology.

On the other hand, Cytochrome P450 is a superfamily of proteins involved in metabolic reactions of activation; however, along with detoxification they are related with metabolic activation of procarcinogens. \textit{CYP2E1} polymorphisms became important when analyzing...
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cytogenetic biomarkers, as this gene is the most abundant in lymphocytes. CYP2E1 c2 allele alters gene expression by elevating protein levels and lowering basal protein activity, when compared with the homozygous c1c1. With respect to both susceptibility and early effect biomarkers, our results are consistent with other studies in which it was established that the c1c1 genotype is associated with increased frequency of chromosomal aberrations in workers exposed to organic solvents. Although no differences have been shown in the basal activity of the two variants of the enzyme (c1 and c2), the c2 variant seems to be more sensitive to damage by interaction with substances as isoniazid, which suggests that c1c1 genotype is less sensitive to damage and therefore explains why it generates more free radicals under the same exposure, which implies an increase in the number of chromosomal alterations that can be expressed in the formation of micronuclei. The presence of micronuclei, on the other hand, is associated with that of nucleoplasmic bridges, as has been shown in previous studies so that the presence of micronuclei increases the risk of nucleoplasmic bridges. Under this situation, the number of micronuclei can be two to ten times greater than that of nucleoplasmic bridges, and up to 100 times greater than that of nuclear buds.

Our study should motivate the strengthening of actions aimed at prevention and risk control by employers and occupational hazard managers to promote more active occupational epidemiological surveillance that seeks to protect the workers. We have identified unacceptable failures in the basic protective measures that should be observed by workers exposed to carcinogenic agents such as the organic solvents contained in car paint. Our results also provide support for the development of more studies to consolidate findings related to the susceptibility effect exerted by the c1c1 allele of the CYP2E1 polymorphism.

Our study had certain limitations due to its methodological design, such as the fact that it only allowed us to establish non-causal associations, and that there could be a memory bias in the workers during data collection with regard to the names of the solvents they used. Workers with more severe symptoms could be more likely to think more carefully and in more detail about the solvents they used compared to those with less severe symptoms. Because workers had to work with solvents for at least 5 years to be included as “exposed” in the study, the variability in recalling the level of exposure among those in the exposed group is not likely to have impacted the results on associations between organic solvents and genotoxic effects.

According to the study results, and in accordance with the recommendations of the 2015 Guideline for Comprehensive Health and Safety in the Workplace for workers exposed to benzene and its derivatives, we would suggest that employers of workers exposed to organic solvents, especially those who work in automotive painting, may educate their employees regarding the harmful effects of these substances, and how to protect themselves through training in safe processes and in the use of personal protective equipment, safety standards, and engineering and administrative controls.

It is worthy to note that, due to the synergistic effect between smoking and exposure to organic solvents, it is necessary to implement strategies that lead to the elimination of this practice and the promotion of healthy lifestyles. Regarding the flaws detected in the use of personal protection, it is important to establish a technical program for the selection and management of approved respirators as well as skin and eye protection, according to the type of exposure that is identified in the workstation.

Our study sample is part of a population that is occupationally exposed to organic solvents that can cause chronic poisoning; therefore, it is imperative to establish occupational epidemiological surveillance programs that include all workers. These programs must be accompanied by periodic evaluations, including complete clinical and occupational history, as well as the blood count and biomarker results recommended by the ACGIH. This will make it possible to detect minor changes due to exposure to organic solvents in a timely manner.
Conclusions

The workers with CYP2E1 polymorphisms, specifically the c1c1 genotype, are exposed to organic solvents, are more likely to have somatic cell mutations, a condition associated with increased susceptibility to diseases such as cancer.

The significant differences in the frequencies of micronucleated cells and nucleoplasmic bridges found between the exposed and the non-exposed, showed a high degree of genotoxicity attributed to the use of organic solvents in the preparation of car paint.

The symptoms included in the Q16 questionnaire were significantly higher in the exposed group, suggesting an associated risk of neurotoxicity and were identified failures in the basic protection measures that exposed workers must have to carcinogens such as organic solvents.

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