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Genotoxic and Hematological Effects in Children Exposed to a Chemical Mixture in a Petrochemical Area in Mexico

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Abstract Children living in Coatzacoalcos, Veracruz, and in nearby surrounding areas are exposed to a mixture of pollutants from different sources. Previous studies in the area have reported genotoxic and haematotoxic compounds, such as lead (Pb), benzene, toluene, and polycyclic aromatic hydrocarbons (PAHs), in environmental and biological samples. The final toxic effects of these compounds are unknown because the toxic behaviour of each compound is modified when in a complex mixture. This is the first study on the exposure and effect of chemical mixtures on children who live near a petrochemical area. The aim of this study was to evaluate genotoxicity and haematological effects in children environmentally exposed to such mixtures and to determine whether the final effect was modified by the composition of the mixture composition. Biomarkers of exposure to Pb, benzene, toluene, and PAHs were quantified in urine and blood samples of 102 children. DNA damage was evaluated using comet assay, and haematological parameters were determined. Our results show that Pb and toluene did not surpass the exposure guidelines; the exposure was similar in all three localities (Allenede, Mundo Nuevo, and López Mateos). In contrast, exposure to PAHs was observed at three levels of exposure: low, medium, and high. The most severe effects of these mixtures were strictly related to coexposure to high levels of PAHs.

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Coatzacoalcos, Veracruz, is a city on the Gulf of Mexico located 7 km from the industrial corridor and where significant chemical and petrochemical activities have been developing since the 1960s. Several sources of toxic compounds, in addition to the contaminant emissions originating from industry, exist and include the following: pesticide and herbicide applications; maritime, vehicular, and railroad traffic; and urban and industrial discharges, among others. As a whole, the current and historical emissions of contaminants at this site have generated a complex mixture of chemical substances within the environment.

Previous studies have reported diverse contaminants in environmental and biological matrices within the area. The list includes benzene and toluene within water samples collected from the Coatzacoalcos river (Stringer et al. 2001; Riojas-Rodriguez et al. 2008) and polycyclic aromatic hydrocarbons (PAHs) and metals (arsenic, lead [Pb], zinc, cadmium, mangenese, nickel, cobalt, copper, chromium, vandium, and mercury) in tissue samples collected from aquatic fauna and sediment (Espinosa-Reyes et al. 2010; Rosales and Carranza 2005; Vázquez-Botello et al. 2004). These compounds create a complex mixture composed of so many chemicals that the composition of the mixture has not been fully characterised either qualitatively or quantitatively; furthermore, the composition of the mixture may vary (ATSDR 2001). Moreover, possible interactions may occur between the substances that comprise the mixture, thus modifying the toxicological behaviour of any single compound and consequently its effects on the exposed population.

In this study area, some compounds, such as Pb, benzene, and PAHs, generate genotoxic effects (Bolin et al. 2006; Böstrom 2002; Mielzyńska et al. 2006; Ruchirawat et al. 2007). In addition, both Pb and benzene generate haematoxicity (Jain et al. 2005; Qu et al. 2002; Schwartz et al. 1990). Moreover, possible interactions may occur among these substances, such as the capacity of toluene to modify the toxicity of benzene (ATSDR 2004). This scenario is complex and represents a health risk for the population living in the area. Children are the most vulnerable to the toxic effects of contaminants (International Programme on Chemical Safety 2006). Concentrations of contaminants lower than those described as harmful for adults may have damaging toxic effects on a child if exposure occurs during a stage of biological vulnerability at which point adverse effects could manifest later in life (USEPA 2006). As previously described, the mixture of contaminants in the study area is capable of generating diverse effects that may affect the healthy development of exposed children.

The aim of this study was to evaluate the exposure of children living in Coatzacoalcos and other localities around the petrochemical area to PAHs, benzene, toluene, and Pb and to assess the possible biological effects on the DNA and haematological systems after chronic exposure to the chemical mixture.

Materials and Methods

Subjects and Sample Collection

The localities that are potentially most exposed due to their proximity to the industrial area are Allende, Mundo Nuevo, and López Mateos. Another source of pollutants is vehicular traffic. López Mateos is located closer to the Pajaritos Complex and its incinerators, the railroad network, and the maritime terminal. For this study, children age 6–12 years from the three localities were included; they were all born and raised in the study area.

A questionnaire was used to gather information on their health status and on any risk factors that might have influenced the biomarker tests for exposure and their effects. A total of 102 children were included after informed consent was obtained from each participant's parent or legal guardian.

The earliest morning urine sample was collected inside a sterile polypropylene container. Two aliquots were acidified using 6 M HCl and stored at 4 °C and then quantified for *trans,trans*-muconic acid (*t,t*-MA), a benzene metabolite, and hipuric acid (HA), a toluene metabolite. Two other aliquots were processed to quantify 1-hydroxypyrene (1-HOP) as a biomarker for exposure to PAHs.

Peripheral blood samples were collected inside tubes containing ethylene diamine tetraacetic acid as an anticlotting agent. These samples were used to quantify blood Pb levels (BLLs) to evaluate haematological parameters and to perform comet assay. This study was approved by the Bioethical Committee at the Medical School of the Universidad Autónoma de San Luis Potosí. Methods that have already been published are indicated by a reference; only relevant modifications are described.

Quantification of *t,t*-MA and HA

The participants were asked to not ingest processed food for at least 24 h before sample collection. This measure was implemented to prevent any interference from ascorbic acid, which is used as a food preservative and can be metabolised into *t,t*-MA (Weaver et al. 2000). The *t,t*-MA levels were determined using the method described by Ducos et al. (1992) with some minor modifications. For quantification, a high-performance liquid chromatography (HPLC) instrument (HP1100; Agilent) with a UV-Vis detector (G1314A) and a C-18 column (Zorbax) was used. The limit of detection was 0.03 mg/L. Standard IRIS ClinCal Recipe (Munich, Germany) calibrator 9969 (5.1 mg/L *t,t*-MA) was used with a 97.7 % recovery rate.

HA quantifications were performed in accordance with National Institute for Occupational Safety and Health 83001 method (2003); the limit of detection for HA was 2 mg/L. Standard certified IRIS ClinCal Recipe 9969 (1.36 g/L AH) was used for quality-control purposes. The recovery rate was 95.5 %.

Quantification of 1-HOP

1-HOP was quantified in accordance with the method described by Kuusimäki et al. (2004). The analyses were performed using an HPLC instrument (HP1100) equipped with a fluorescence detector (G1321A). The precolumn used was a Zorbax SB-C18, and the column was a Zorbax Eclipse XDB-C18. Under these conditions, the limit of detection for urine was 1.0 nmol/L. A standard certified IRIS ClinCal Recipe 8867 (15.6 nmol/L 1-HOP) was used for quality control purposes with a 95.3 % recovery rate.

Urine Cotinine Assessment

Cotinine levels were determined using Accutest NicAlert stripes (Jant Pharmaceuticals, Encino, CA). A level ≥ 3 outcome for the NicAlert (100–200 ng/mL) indicates either passive or active exposure to tobacco smoke.

Blood Pb Quantification

BLLs were quantified using the method described by Subramanian (1989). The samples were analysed using a

Perkin-Elmer 3110 graphite furnace atomic absorbance spectrophotometer in the presence of a matrix modifier (ammonium phosphate [Triton X-100 with 0.2 % nitric acid]). The percentage of recovery was 96.8 % using a sample provided by the Centers for Disease Control (CDC).

Nutritional Evaluation

Data on the size, weight, and age of the children were collected. The *z*-score for weight-for-age (WAZ) and z-score for length/height-for-age (HAZ) were calculated using the Epi Info 6.0 program (CDC), which uses as a reference the data collected by the National Center for Health Statistics for chronic and acute malnutrition markers. Values were classified according to the CDC criteria: WAZ or HAz-score <-2 indicated that the children were malnourished, whereas WAZ or HAz-score >2 indicated that the children were overweight (CDC 2002).

Comet Assay

DNA damage was assessed through comet assay performed under alkaline conditions as previously described by Singh et al. (1988). The basal level of DNA damage in leukocytes was analysed in 100 cells (50 randomly selected cell nuclei by duplication/individual) and examined using an epifluorescence microscope (Nikon Eclipse E400). The olive tail moment (OTM) was determined through imaging analysis using Komet software, version 4 (Kinetic Imaging, Liverpool, UK).

Blood Cell Counts

Peripheral blood samples were processed 3 h after they had been collected using ADVIA 120 Bayer Coulter T540 equipment. Total white blood cell count (WBC), haemoglobin (HB), haematocrit (HCT), erythrocyte count (ER), and platelet count were determined.

Categories of Exposure

The data collected from each biomarker were grouped into four distinct categories. For each case, the reported levels were handled according to their associated effects and the level of occupational exposure in adults or infants. The categories are listed in Table 1.

Statistical Analysis

The data were transformed logarithmically, with the exception of the nutritional parameters, to obtain a normal distribution. Analysis of variance (ANOVA) tests for the exposure and effect biomarkers were performed at each site, and *post hoc* test (least significant difference [LSD]) was applied if the results were statistically significant. Partial correlations were calculated and adjusted using nutritional parameters (WAZ and HAZ). The statistical analyses were performed using Statistica software version 6.0 (StatSoft, Tulsa, Oklahoma, USA).

Results and Discussion

Population Features and Nutritional Status

The results demonstrate that according to the nutritional parameters (CDC 2002), the population shows no signs of malnutrition, and no significant difference was observed across parameters in all three areas. The low cotinine levels in urine and on the questionnaire, which showed only a 7 % exposure to tobacco smoke, indicated that passive smoking is not a risk factor.

Exposure Assessment

The children evaluated in this study were exposed to a mixture of pollutants from different sources. The results obtained from the exposure evaluations for Pb, PAHs, benzene, and toluene were grouped by location and are listed in Table 2. There was no significant difference across sites in relation to BLLs and *t,t*-MA or HA concentrations.

After analysing all of the data from all of the children, a partial correlation was detected (adjusted through WAZ and HAZ) between *t,t*-MA and HA ($r = 0.24$; $p < 0.05$) and between BLLs and *t,t*-MA levels ($r = 0.23$; $p < 0.05$). These correlations indicate that there might be a source and possible route that acts as a common ground for Pb, benzene, and toluene. These contaminants might come from industrial emissions because both benzene and toluene are produced as raw materials by some industries in the area.

The results of 1-OHP urinary concentrations showed three levels of exposure (Table 2). In Allende, the lowest concentrations were detected (geometric media [GM] 0.20 μmol/mol creatinine [Cr]); the results in Mundo Nuevo showed medium levels (GM 0.41 μmol/mol Cr); and in López Mateos, the highest 1-HOP levels were recorded (GM 1.26 μmol/mol Cr). A significant difference was observed with respect to the other two sites (*post hoc* LSD test $p < 0.001$). These differences in exposure to PAHs suggest that the sources may be different for each location. The common source in the area was vehicular traffic, and another source coupled with vehicular traffic may be industrial emissions due to the city's close proximity to an industrial complex. In López Mateos, the high exposure to PAHs could be a result of its proximity to the Pajaritos

Table 1 Categories of exposure for each biomarker evaluated in the studied population

Biomarker	Category	Levels	Criteria	Reference
BLL (μg/dL)	1	ND <5	Neurologic effects are present, although no biochemical altered parameters were reported in children	Ahamed et al. (2005); Jin et al. (2006)
	2	≥5 to <10	Alterations to δ-aminolevulinic acid dehydratase enzyme activity as well as other enzymes	Ahamed et al. (2005)
	3	≥10 to <20	Concentrations above the CDC intervention level	CDC (1991)
	4	≥20	Levels associated to moderate and severe child anemia as well as a diminished metabolism of vitamin D	Ahamed et al. (2008)
t,t-MA (μg/g Cr)	1	ND <100	Threshold found in children living in rural areas	Amodio-Cocchieri et al. (2001); Ruchirawat et al. (2006)
	2	≥100 to <170	Levels lower than those reported in children living in urban areas	
	3	≥170 to <500	Concentrations lower than the BEI (500 μg/g Cr) proposed by ACGIH and the Official Mexican Norm for occupationally exposed adults	ACGIH (2005); NOM-047-SSA1-2002
	4	≥500	Values greater then BEI value	
HA (g/g Cr)	1	ND <0.36	Concentrations lower than those reported as basal levels for adult nonsmokers	Siqueria and Paiva (2002)
	2	≥0.36 to <0.52	Smokers who have not been exposed occupationally	Roma-Torres et al. (2006)
	3	≥0.52 to <1.6	Concentrations lower than BEI (1.6 g/g Cr) proposed by ACGIH and the Official Mexican Norm	ACGIH (2005); NOM-047-SSA1-2002
	4	≥1.6	Greater than occupationally cut-off points	
1-OHP (μmol/mol Cr)	1	ND <0.24	Lower than concentrations from individuals not being exposed and nonsmokers	European Guide For Occupational Exposure proposed by Jongeneelen (2001)
	2	≥0.24 to <0.76	Below of the cutoff for individuals not being exposed but who are smokers	
	3	≥0.76 to <1.4	Lower than NOAEL for genotoxicity	
	4	≥1.4	Greater than NOAEL	

ND Not detected, *ACGIH* American Conference of Governmental Industrial Hygienists

Complex and its incinerators, the railroad network, and the maritime terminal.

Table 3 lists the percentages of each group of children by category for individual biomarkers according to the cut-offs described in Table 1. BLLs across all three sites were found to be lower than the CDC intervention level (10 μg/dL) for 95 % of the children. However, a significant number of children were classified in the second category (≥5 μg/dL), which is related to some of the adverse effects. BLLs in all children were detected to be above the average levels reported by the National Health and Nutrition Examination Survey (NHANES) (2009) in children age 6–11 years (1.25 μg/dL).

In contrast, in all three localities, concentrations of *t,t*-MA were greater than the basal levels reported for adults in 70–80 % of the children, whereas 30 % were greater than the BEI point (500 μg/g Cr). Urinary *t,t*-MA in children living in the study area were similar to values reported in adults who were occupationally exposed to 1 ppm of benzene in the air from chemical industries (Waidyanatha et al. 2004).

In addition, 50 % of the urinary HA concentrations in this population evaluated were lower than the cut-off for basal levels (≤0.36 g/g Cr), which indicates exposure to toluene; Mundo Nuevo had the highest levels of HA, although the result was not statistically significant.

Urinary 1-OHP concentrations were shown to reflect three levels of exposure: low, medium and high. However, 1-OHP concentrations detected across all three locations were greater than the NHANES GM for the group of children age 6–11 (approximately 0.12 μmol/mol Cr) found by some other studies (Ruchirawat et al. 2007). In accordance with the pre-established cut-off points, Allende had the lowest exposure levels: In this community, 89 % of the children were classified in the first two categories. Mundo Nuevo showed an intermediate level of exposure; 65 % of the children showed values similar to adults nonoccupationally exposed to PAHs, although almost 24 %

Table 2 BLLs and quantified urine metabolites in the studied population

Locations	Allende	Mundo Nuevo	López Mateos
BLL (μg/dL)	4.5 (2.5–10.1)[a]	5.2 (2.5–10.4)	4.6 (2.9–9.6)
	$n = 45$	$n = 37$	$n = 20$
t,t-MA (μg/g Cr)	388 (44–1784)	363 (63–5521)	369 (62–1414)
	$n = 46$	$n = 38$	$n = 21$
HA (g/g Cr)	0.34 (0.03–1.98)	0.46 (0.09–1.5)	0.38 (0.06–2.12)
	$n = 46$	$n = 38$	$n = 21$
1-HOP (μmol/mol Cr)	0.20 (0.04–2.08)	0.41 (0.07–4.04)	1.26 (0.78–3.05)*
	$n = 44$	$n = 34$	$n = 16$

[a] Geometric mean (minimum-maximum)

* Significantly different from the rest of the evaluated locations using *post hoc* test LSD ($p < 0.001$)

Table 3 Percentages of children for each category of biomarker of exposure ($n = 91$)

	Allende $n = 41$				Mundo Nuevo $n = 37$				López Mateos $n = 16$			
Categories	1	2	3	4	1	2	3	4	1	2	3	4
BLL	65.1	30.5	4.4	0	44.8	49.9	2.6	0	66.7	33.3	0	0
t,t-MA	4.4	4.4	58.6	34.8	10.5	11.5	43.8	34.2	9.5	0	57.2	33.3
HA	43.5	30.4	19.6	6.5	28.9	31.6	39.5	0	42.9	33.3	12	4.8
1-HOP	67.4	21.7	2.2	4.7	31.6	39.4	5.2	23.8	0	0	57.2	42.8

exceeded the nonobservable adverse effect level (NOAEL) (1.4 μmol/mol Cr). The data showed that the children in López Mateos had the highest exposure to PAHs. Furthermore, there were no urinary 1-HOP levels similar to individuals who were not exposed occupationally, and 42 % of the children's levels exceeded the NOAEL. Martínez-Salinas et al. (2009) reported urinary 1-OHP levels in children exposed to PAHs from different sources. Compared with this study, 1-OHP concentrations in children from Allende were similar to the values found in children exposed to emissions of heavy vehicular traffic. Mundo Nuevo showed levels similar to those of children living near brick kilns. In López Mateos, all 1-OHP values were greater than those of smokers who were not occupationally exposed but lower than concentrations reported in children exposed to biomass combustion (2.2 μmol/mol Cr).

Genotoxicity Evaluation

Table 4 lists the results obtained from the evaluation of DNA damage. Genotoxicity results in all three locations showed that the OTM values were two or even three times greater than the basal value reported by Bajpayee et al. (2002). Allende had the lowest DNA damage and was significantly different from that of the other two communities (*post hoc* LSD test; $p < 0.001$); López Mateos showed the highest DNA damage. These findings may be associated with PAH-exposure levels; in fact, a significant partial correlation between OTM and urinary 1-HOP levels ($r = 0.24$; $p < 0.05$ [WAZ and HAZ adjusted]) was obtained. This same correlation between 1-HOP levels and genotoxicity has been previously discussed in other studies (Ruchirawat et al. 2007; Mielzyńska et al. 2006).

Table 4 OTM mean comparison and percentages of children with values greater then the basal level in each location

Location	Allende	Mundo Nuevo	López Mateos
OTM	8.3 (3.1–16.8)[a]*	10.6 (5.6–22.9)	11.7 (7.4–15.9)
	$n = 44$	$n = 37$	$n = 16$
% >4[b]	95.5	100	100

[a] Geometric mean (minimum-maximum)

[b] Moment = 4 (basal value established by Bajpayee et al. (2002))

* Significantly different from the rest of the locations ($p < 0.001$) by *post hoc* LSD test

However, this severe damage could be explained if we assume that it might be a result of simultaneous exposure to several genotoxic compounds in a mixture, such as a combination including benzene, which was also found to have a high exposure in this area. This coexposure might represent a major genotoxic risk because interactions between benzene and PAHs may then occur. Both of these compounds are metabolised by CYP450, which generates metabolites capable of producing oxidative stress, adducts, and mutagenesis (Shimada 2006; Snyder 2000). Arayasiri et al. (2010) evaluated two scenarios for benzene exposure in four susceptible groups coexposed to PAHs: 8-hydroxy-2'-deoxyguanosine (8-OHdG), DNA strand breaks, and DNA repair capacity were measured as biomarkers of the

Table 5 Pearson correlation coefficients between *t,t*-MA and BLL concentrations and haematologic parameters in López Mateos ($n = 20$)

	Not adjusted	Adjusted[a]
t,t-MA *vs.*		
HB	–0.62**	–0.60**
HCT	–0.65*	–0.64**
WBC	–0.38	–0.51*
RBC	–0.47*	–0.42*
BLLs vs.		
WBC	–0.53*	–0.69*

* $p < 0.05$

** $p < 0.01$

[a] Adjusted by height according to HAZ and WAZ

early effects of exposure to carcinogenic compounds. The results obtained in Arayasiri's study showed that high exposure to PAHs might have influenced the 8-OHdG levels, DNA strand breaks, and DNA repair capacity. Unfortunately, our results for benzene exposure cannot demonstrate such interactions because the observed *t,t*-MA concentrations did not make up an exposure gradient.

Haematoxicity Evaluation

No significant differences were found for the mean haematological parameters evaluated across locations. However, when the haematological parameters and the exposure biomarkers were correlated, only the correlation between *t,t*-MA and HCT for the whole population data were found to be significant ($r = -0.2$; $p < 0.05$). Partial correlations were calculated for each locality, but these correlations were only significant in López Mateos. Table 5 lists the negative correlations between exposure to benzene; between HB and HCT ($p < 0.001$), WBC, and WBC ($p < 0.05$); and between BLLs and WBC ($p < 0.05$); these were all obtained in López Mateos. These correlations are greater than those previously reported by Qu et al. (2002) for workers exposed to aerial low benzene concentrations (2.26 mg/L). In those studies, the researchers reported an association between the levels of *t,t*-MA with RBC ($r = -0.26$) and with WBC ($r = -0.14$). Neither Qu et al. nor any other studies show evidence of any clinical effects associated with chronic exposure to benzene at low concentrations in occupationally exposed individuals (Collins et al. 1991; Lan et al. 2004).

This strong association between the decreased haematological parameters and benzene exposure, which was only found in Lopez Mateos, might be explained through the effects of coexposure to benzene and high-level PAHs. These effects may account for a greater sensitivity to the adverse effects of benzene. This reasoning is plausible if one takes into account that one of the toxic mechanisms of some PAHs, such as benzo(*a*)pyrene, is their high affinity for aryl hydrocarbon receptor (AhR) (Bin et al. 2008; Stevens et al. 2009). Many studies indicate that AhR plays an important role in benzene haematoxicity, positing two possible mechanisms by which benzene-induced hematopoietic toxicity may be regulated by AhR activation. First, it is possible that the altered expression of enzymes by AhR, which is responsible for the production of toxic metabolites, and/or the activation of the AhR by benzene metabolites could contribute to differential susceptibility to benzene (Hirabayashi et al. 2004; Yoon et al. 2002) Second, some theories exist that the altered presence and/or activity of AhR could modulate the responses of haematopoietic stem/progenitor cells to benzene, specifically in controlling the balance between quiescence and proliferation in haematopoietic stem cells (Gasiewicz et al. 2009; Hirabayashi et al. 2008).

In contrast, the results obtained in this study show a significant negative correlation between BLLs and WBC counts in López Mateos; this finding is remarkable because the effects of a haematotoxicity for BLLs < 10 μg/dL have not been proven. However, Mielzyńska et al. (2006) associated coexposure to PAHs with an increase in micronuclei of peripheral lymphocytes in children with BLLs < 10 μg/dL compared with children exposed only to low Pb levels. Therefore, we also consider it important to evaluate the role of Pb when considering final haematotoxic effects.

Conclusion

Children are more susceptible to the toxicity of contaminants than adults, but the risk of late-effect development further increases when children are exposed to a mixture of concentrations for a long period of time, even if the toxins within the mixture do not surpass the safety levels stated for each compound. This condition of biologic-mixture time vulnerability may generate different toxic effects different from those expected after exposure to a single contaminant, even resulting in adverse effects long after exposure (USEPA 2006).

The children evaluated in this study were exposed to a mixture of pollutants from different sources. Our results suggest that both the genotoxicity and haematological effects associated with exposure to low levels of benzene and Pb are enhanced by coexposure to PAHs at high levels.

However, the children in the Coatzacoalcos petrochemical area may be responding to other adverse effects that we did not consider; for example, the BLLs detected in this study are associated with neurotoxicity. The final toxic effect may very well be a result of interactions with other

compounds present in the area that were not quantified for the purposes of this project, such as DDT, dioxins, vinyl chloride, etc. Therefore, further studies on this particular issue are necessary.

Finally, this study shows that it is important to address environmental safety with an open mind because possible interactions may result from simultaneous exposure to toxic agents, even if they occur individually at concentrations considered to be safe. This exposure to mixed pollutants adds to the susceptibility of the affected individuals and generates a long-term negative effect due to the continuous stress on the organism struggling to reach a homeostatic state. In conclusion, real solutions are required to decrease or eliminate the adverse effects of mixed pollutants as much as possible.

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