CYP2E1 epigenetic regulation in chronic, low-level toluene exposure: Relationship with oxidative stress and smoking habit

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ABSTRACT

Background: CYP2E1 is a versatile phase I drug-metabolizing enzyme responsible for the biotransformation of most volatile organic compounds, including toluene. Human toluene exposure increases CYP2E1 mRNA and modifies its activity in leucocytes; however, epigenetic implications of this interaction have not been investigated.
Goal: To determine promoter methylation of *CYP2E1* and other genes known to be affected by toluene exposure.
Methods: We obtained venous blood from 24 tannery workers exposed to toluene (mean levels: $10.86 +/- 7$ mg/m^3) and 24 administrative workers (reference group, mean levels $0.21 +/- 0.02$ mg/m^3) all of them from the city of León, Guanajuato, México. After DNA extraction and bisulfite treatment, we performed PCR-pyrosequencing in order to measure methylation levels at promoter region of 13 genes.
Results: In exposed group we found significant correlations between toluene airborne levels and *CYP2E1* promoter methylation (r $= -.36$, $p < 0.05$), as well as for *IL6* promoter methylation levels (r $= .44$, $p < 0.05$). Moreover, *CYP2E1* promoter methylation levels where higher in toluene-exposed smokers compared to nonsmokers ($p = 0.009$). We also observed significant correlations for *CYP2E1* promoter methylation with *GSTP1* and *SOD1* promoter methylation levels (r $= -.37$, $p < 0.05$ and r $= -.34$, $p < 0.05$ respectively).
Conclusion: These results highlight the importance of considering *CYP2E1* epigenetic modifications, as well as its interactions with other genes, as key factors for unraveling the sub cellular mechanisms of toxicity exerted by oxidative stress, which can initiate disease process in chronic, low-level toluene exposure. People co-exposed to toluene and tobacco smoke are in higher risk due to a possible CYP2E1 repression.

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Introduction

Human toluene exposure has been related with peripheral and central neurotoxicity (Shih et al., 2011; Gupta et al., 2011), nephrotoxicity (González-Yebra et al., 2006; Voss et al., 2005), respiratory system disease (Al-Batanony et al., 2012; Yoon et al., 2010), immune system alterations (Haro-García et al., 2012; Bulog et al., 2011) and effects on reproductive system (Hannigan and Bowen, 2010; Bukowski, 2001). Some of these studies found adverse toluene effects not only in low-level occupational exposure but also in individuals living at home where indoor, chronic toluene exposure has been observed. Molecular basis of adverse health effects from toluene exposure in humans are not fully elucidated. Human and cell-culture studies have implicated oxidative stress, inflammation and induction of apoptosis in toluene induced toxicity (Haro-García et al., 2012; Sarma et al., 2011; Mögel et al., 2011; Kim et al., 2011). Toluene has also shown genotoxic effects (Moro

et al., 2012; Cassini et al., 2011; Rekhadevi et al., 2010; González-Yebra et al., 2009) and an increased risk of developing hematologic malignancies (Breccia et al., 2012; Cocco et al., 2010; Costantini et al., 2008; Jung et al., 2007). Toluene biotransformation takes place in the liver by the cytochrome P450 2E1 (CYP2E1) isoform, producing mainly hippuric acid, which is excreted in urine (Nakajima and Wang, 1994). Toluene also up-regulates its own metabolism by inducing CYP2E1 activity in rat liver and peripheral lymphocytes (Gonzalez-Jasso et al., 2003). CYP2E1 induction is relevant because this enzyme activates many xenobiotics to become ultimate toxicants (Guengerich and Shimada, 1991). In fact, CYP2E1 induction may be the first step leading to chemical induced carcinogenesis (Lieber, 1997). Also, CYP2E1 mRNA levels in peripheral lymphocytes correlated with exposure levels of toluene in printing industry workers (Mendoza-Cantú et al., 2006). In low-level toluene exposure, CYP2E1 enzymatic activity is reduced and was linked with cumulative exposure time and body mass index (Jiménez-Garza et al., 2012). On the other hand, tobacco smoke and nicotine in humans have also been shown to activate CYP2E1 expression (Benowitz et al., 2003; Joshi and Tyndale, 2006; Al-Arifi et al., 2012). A recent study in

animals reported toxic reproductive effects associated with tobacco smoke-induced CYP2E1 activation with a consequent increase in oxidative stress and macromolecular adducts (Sobinoff et al., 2013).

When studying early effects derived from chemical exposures, such as direct DNA biochemical modifications, methylation could be a key factor to understand the triggering of several cascades related to gene expression involved in pathologic cellular events (Szyf, 2011). Epigenetic changes like DNA methylation have come a long way in order to unravel the interactions of DNA with other molecules from endogenous origin. By investigating covalent, reversible modifications, in this case the addition of a methyl group in cytosine number 5 in Cytosine–Guanine dinucleotides (CpGs) in the DNA sequence, (either global or gene-specific), this field of knowledge is of high importance as an effort to explain gene-expression regulation at the chromatin level (Watson and Goodman, 2002). One of the most accepted roles of *de novo* DNA methylation, specifically in promoter regions with high CpG density, is gene silencing (Hackett and Surani, 2013). In support of this assumption, experiments carried out on *in vitro* models derived from different tissues, as well as in postmortem human tissues, have reported a negative correlation between gene expression and promoter methylation in cancer cells (Jia and Salvatore, 2013) and also in non-tumorigenic cells (Gordon et al., 2014; Kaut et al., 2012).

Aberrant methylation is a hallmark of cancer cells, and therefore epigenetic effects of environmental carcinogens have been intensively investigated. Among volatile organic compounds, benzene, a known carcinogen, has shown contradictory results for induction of p15 tumor suppressor gene methylation, including reports on high and low methylation (Bollati et al., 2007; Seow et al., 2012, respectively). More recent reports have described higher methylation in promoter area of genes related to DNA repair and hematotoxicity as a result of benzene exposure (Xing et al., 2013; Li et al., 2013). In spite that there have been identified molecular pathways affected by toluene exposure in human cells, there is still a knowledge gap on whether chronic exposure to toluene can induce epigenetic alterations. In the present study, we try to fill this knowledge gap by investigating DNA methylation in the promoter regions of 13 genes related with inflammation, oxidative stress, DNA repair and xenobiotic metabolism in leukocytes from Mexican tannery workers exposed to toluene.

Methods

Study participants and ethical approvals. As previously reported (Jiménez-Garza et al., 2012), after agreement with owners of the factory, workers from two different tanneries in the city of León, Guanajuato, México were invited to participate in the study. In their workplaces, workers perform a specific process of tanning, known as "finishing" which has an extensive volatile organic compounds (VOCs) use. Both tanneries differed substantially from each other; one was very traditional (i.e., housed in an old building, with very limited industrial hygiene measures, tannery A), while the other was in a modern building designed specifically for industrial use (tannery B). Administrative personnel of two universities were also invited to participate as non-exposed controls. We obtained informed consent from 27 participants (all males) from the tanneries (exposed group, E) and 27 participants (all males) from the universities (control group, NE). For E group, we obtained informed consent from 10 persons in tannery A and 17 persons from tannery B. The study was approved by the Ethics Committee of the participating institutions.

Individual questionnaire and body mass index (BMI). Demographical, behavioral and health data were obtained through an individual questionnaire; also a brief medical history was performed. All participants included were clinically healthy. After questionnaire was finished, participants were weighed and measured according to standard procedures and BMI was calculated (Wellens et al., 1996).

Individual exposure level determination. Before work shift started, a personal passive diffusive monitor (3M, 3510 model, St Paul MN, USA) was attached to each person's clothes at the respiratory tract level and carried throughout the working period. At the end of work shift, after 8 h, each monitor was retired and packed in the sealed container provided by the manufacturer. Once packed, all diffusers were sent for analysis by gas chromatography/mass spectrometry (GC/MS) to the Braun Intertec Hygiene Industrial Laboratory (Minneapolis, MN, USA). We asked exposure levels for benzene, toluene and ethylbenzene.

Sample preparation. At the workplace, we obtained 4 ml peripheral blood in EDTA tubes from each participant. Out of 27 persons initially invited, we obtained blood samples from 24 per group (exposed and nonexposed; from E group: 9 samples from tannery "A" and 15 samples from tannery "B"). Samples were transported to laboratory and stored at −80 °C until further analysis. After thawing on ice, we aliquoted 200 μl whole blood from each participant for genomic DNA isolation using the DNA Blood and tissue kit (Qiagen, Valencia, CA, USA). DNA concentration was measured on a nanophotometer (IMPLEN, Germany). Extracted DNA was stored at −80 °C for subsequent analysis.

DNA methylation analysis. 500 ng genomic DNA was bisulfite-treated using the EZ-96 DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Final elution was performed with 30 μl M-Elution Buffer. Bisulfite-treated DNA was aliquoted and stored at −80 °C until further use. We performed DNA methylation analyses on bisulfite-treated DNA by highly quantitative PCR-pyrosequencing technology. The PCR and pyrosequencing primer sequences for *COX-2*, *CYP2E1*, *GSTM1*, *GSTP1*, *HMOX-1*, *IL6*, *iNOS*, *p53*, *RAD51*, *SOD1*, *TNFα*, *TOP2A*, and *WRAP5*. The PCR and sequencing primers are showed in Supplementary Table 1. PCR amplification was performed at standard conditions using the GoTaqW Hot Start Polymerase (Promega, Madison, WI). We used a PSQ Q96 MD pyrosequencing System (QIAGEN, Valencia, CA), as previously described (Byun et al., 2013). In brief, bisulfite-treated DNA (50 ng) was amplified with a biotin-labeled primer in a PCR reaction, which enables the conversion of the PCR product to a single-stranded DNA template suitable for pyrosequencing. Specificity of PCR amplicons was assessed by 2% agarose gel analysis. Pyrosequencing was carried out using the PyroMark Q96MD System (Qiagen) according to manufacturer's protocol.

The percentage of methylation (%5-mC) at each CpG site was expressed as the level of methylated cytosine divided by the sum of methylated and unmethylated cytosines. To estimate bisulfite conversion efficiency, we placed genomic DNA samples by duplicate on each bisulfite conversion plate to estimate the internal plate variation of bisulfite conversion and the pyrosequencing reaction, as a quality control check. We also added universal PCR products amplified from cell-line DNA on each pyrosequencing plate to check plate-to-plate and run-to-run variation. In addition, the Pyrogram peak pattern was visually inspected to confirm the quality of the reaction in every sample.

Statistical analysis. Data normality was assessed by Shapiro–Wilk test. Groups were compared (exposed vs. nonexposed) either by Mann–Whitney U test or by Student's t test, based on data normality. We also performed simple regression analysis for methylation levels and other variables such as age, BMI, exposure levels, cumulative time of exposure as well as between methylation levels in different genes. IBM SPSS 19™ software was used for this analysis. In addition, we performed a multiple linear regression analysis of the methylation levels for each CpG studied; we included as covariates: age, BMI, toluene exposure levels, smoking status, cumulative time of exposure and place of labor (tannery A or B). Significance was set at $p < 0.05$. Stata version 11 software (StataCorp LP, College Station, TX) was used. We performed an additional diagnostic analysis (outliers' influence, normality of residuals, linearity, and homoscedasticity), in all those models showing statistical significance.

Results

Characteristics of the study participants

A summary of characteristics of the study participants in E and NE group are shown in Table 1. Relevant biological variables such as age and BMI were similar in both groups ($p = 0.86$ and $p = 0.82$ for age and BMI, respectively according to Student's t test results). When analyzing differences between subgroups in the E group (tannery A vs. tannery B), variables age, and cumulative time of exposure did not show significant differences. BMI showed a trend ($p = 0.05$) between tanneries, with higher values for tannery B participants.

VOC exposure levels

Mean toluene levels were higher in the exposed workers (4.3 to 38.1 mg/m^3) compared to control group. Exposure levels were lower than the Threshold Limit Value − Time Weighted Average (TLV-TWA) established by national and international agencies in all cases (Table 2). There was no statistically significant difference for benzene and ethylbenzene exposure levels between groups (Table 2). Toluene exposure levels, the only solvent studied showing significant differences between groups, did not show statistical significant differences between E subgroups (tannery A vs. tannery B) (Table 2).

Promoter methylation levels and its correlation with study covariates

Initially 13 genes were selected for their promoter methylation analysis. After pyrosequencing run, duplicates which had Pearson correlation coefficient < 0.3 (runs for p53, RAD51, GSTM1 and WRAP53) were discarded from the further analysis. Promoter methylation of the following genes was included in the final analysis: COX-2, CYP2E1, GSTP1, HMOX-1, IL-6, iNOS, SOD1, TNFα and TOP2A at least in one methylation position.

In Table 3 we give result of simple linear regression between methylation levels and study covariates in both exposed and control groups. We also checked the association of CpG sites in exposed group with the exposure parameters i.e., levels of exposure and cumulative time of

Table 2
Individual exposure levels (mg/m^3) for the VOCs studied in exposed (E) and nonexposed (NE) groups. Toluene exposure levels for E subgroups (tannery A and tannery B) are included.

VOC		Mean	SD	Median	Minimum	Maximum
Benzene	NE	0.19	0.01	0.18	0.16	0.24
	E	0.19	0.06	0.16	0.16	0.36
Ethylbenzene	NE	0.24	0.02	0.23	0.21	0.31
	E	0.25	0.12	0.21	0.21	0.79
Toluene	NE	0.21	0.02	0.21	0.18	0.27
	E	10.8	7.5	8.4	4.3	38.1*
	Tannery A	7.72	3.2	7.95	1.92	12.7
	Tannery B	12.76	8.7	9.69	5.28	38.1

* $p < 0.001$.

exposure. Results with statistical significance (p value < 0.05) are shown in the table.

As shown in Table 3, a number of CpG sites showed statistical significance or borderline significance with exposure variables and study covariates for exposed group. Such cases corresponded to GSTP1, iNOS, and IL6 genes. We then pooled the values obtained for all the positions and found that these values were correlated with important variables from the study population (age, BMI and exposure levels, respectively). In the case of CYP2E1, only one CpG (methylation position number 2) from the five studied showed a statistical significance (all correlations shown in Fig. 1). After performing a secondary analysis using a general linear model with covariates, we confirmed that age, BMI, and toluene exposure levels showed a significant association with the methylation status in three promoter regions (GSTP1, iNOS, and IL6 respectively). With this secondary analysis, we ruled out that other covariates (independent variables) could be affecting the Methylation status of these three promoters (dependent variables). For CYP2E1 methylation in all positions only position 2, had a significant relationship with toluene exposure levels, however, categorical values such as "tannery" (A or B) and also "current smoking status" (Yes or no) also showed a significant relationship ($p < 0.05$) for all positions studied (data not shown).

Differences between CYP2E1 promoter methylation levels in subgroups: exposed smokers and workers from different tanneries

As mentioned above, while performing the multiple linear regression analysis with covariates in each of the five positions analyzed for CYP2E1 promoter, we noticed that categorical variables "smoker" and "tannery" showed significant associations for all positions. Because of this, we performed a separated analysis comparing mean methylation levels (averaging methylation values obtained from the five positions studied) in both tanneries, as well as in exposed participants identified as current smokers. Methylation levels were higher in exposed smokers (Mean 29.6 +/− 10.8) compared to nonsmokers (Mean 14.8 +/− 8.2; $p < 0.005$, Fig. 2A); also, methylation levels were higher for tannery A participants (Mean 31.7 +/−7.9) compared to tannery B (Mean 17.4 +/− 12.9; $p < 0.05$, Fig. 2B). Since in tannery A seven participants out of a total of nine were identified as current smokers (Table 1), in Fig. 2C we show CYP2E1 methylation value distribution for smokers separated by tannery.

Correlations between methylation levels for different genes in the exposed group

In order to explore possible associations in the methylation levels between the 9 genes studied, we performed simple lineal regression comparing each methylation position in the promoter region studied between genes. We found statistical significant correlations for all positions (five) in the CYP2E1 promoter with position 1 in the GSTP1

Table 1

Variable	Group and subgroup[a]	Mean	SD	Median	Minimum	Maximum
Age (years)						
	E	36.7	13.8	33.5	19	63
	NE	35.9	12.9	33	19	66
	Tannery A	39.4	14.1	38	23	63
	Tannery B	35.07	13.8	33	19	62
Body mass index (kg/m^2)						
	E	25.3	3.2	24.7	19.3	32
	NE	26.09	4.03	25.01	18.8	35.2
	Tannery A	23.7	2.4	24.6	19.3	24.8
	Tannery B	26.2	3.4	27.5	19.7	32.09
Cumulative occupational exposure time (months)						
	E	67.8	68.1	45	2.5	300
	NE	NA	NA	NA	NA	NA
	Tannery A	52	32.1	42	18	120
	Tannery B	68.6	83.7	24	6	300
Current smokers						
	E	n = 13				
	NE	n = 3				
	Tannery A	n = 7				
	Tannery B	n = 6				

NA = Not applicable.
[a] n = 24 per group; n = 9 for tannery A; n = for tannery B.

Table 3
Correlations between relevant biological and exposure variables with promoter methylation in the groups studied.

| Group	Dependent variable	Independent variable	Pearson correlation coefficient	p value
Nonexposed				
	SOD1 position 4 methylation levels	*Age*	−.16	0.22
	GSTP1 position 1 methylation levels	*Age*	.11	0.3
	GSTP1 position 3 methylation levels	*Age*	.16	0.22
	iNOS position 1 methylation levels	*BMI*	.05	0.4
	iNOS position 2 methylation levels	*BMI*	.4	0.02[a]
	TNFa position 4 methylation levels	*BMI*	−.02	0.45
Exposed				
	SOD1 position 4 methylation levels	Age	.39	0.03[a]
	GSTP1 position 1 methylation levels	Age	−.32	0.06
	GSTP1 position 3 methylation levels	Age	−.34	0.04[a]
	iNOS position 1 methylation levels	BMI	.37	0.03[a]
	iNOS position 2 methylation levels	BMI	.34	0.04[a]
	TNFa position 4 methylation levels	BMI	−.55	0.003[a]
	IL6 position 2 methylation levels	Cumulative time of exposure (Months)	.38	0.03[a]
	GSTP1 position 3 methylation levels	Toluene exposure levels	.37	0.03[a]
	SOD1 position 3 methylation levels	Toluene exposure levels	.36	0.04[a]
	IL6 position 1 methylation levels	Toluene exposure levels	.39	0.02[a]
	IL6 position 2 methylation levels	Toluene exposure levels	.44	0.01[a]
	CYP2E1 position 1 methylation levels	Toluene exposure levels	−.3	0.07
	CYP2E1 position 2 methylation levels	Toluene exposure levels	−.36	0.04[a]
	CYP2E1 position 3 methylation levels	Toluene exposure levels	−.3	0.07
	CYP2E1 position 4 methylation levels	Toluene exposure levels	−.3	0.07
	CYP2E1 position 5 methylation levels	Toluene exposure levels	−.3	0.07

[a] Statistically significant.

promoter. After averaging all methylation values found in the five different positions of the *CYP2E1* promoter region, the regression analysis with position 1 in the *GSTP1* promoter region corroborated the negative relation (Fig. 3 panel A). Another negative correlation was found between position 2 of the *CYP2E1* promoter and position 3 of the *SOD1* promoter region (Fig. 3 panel B).
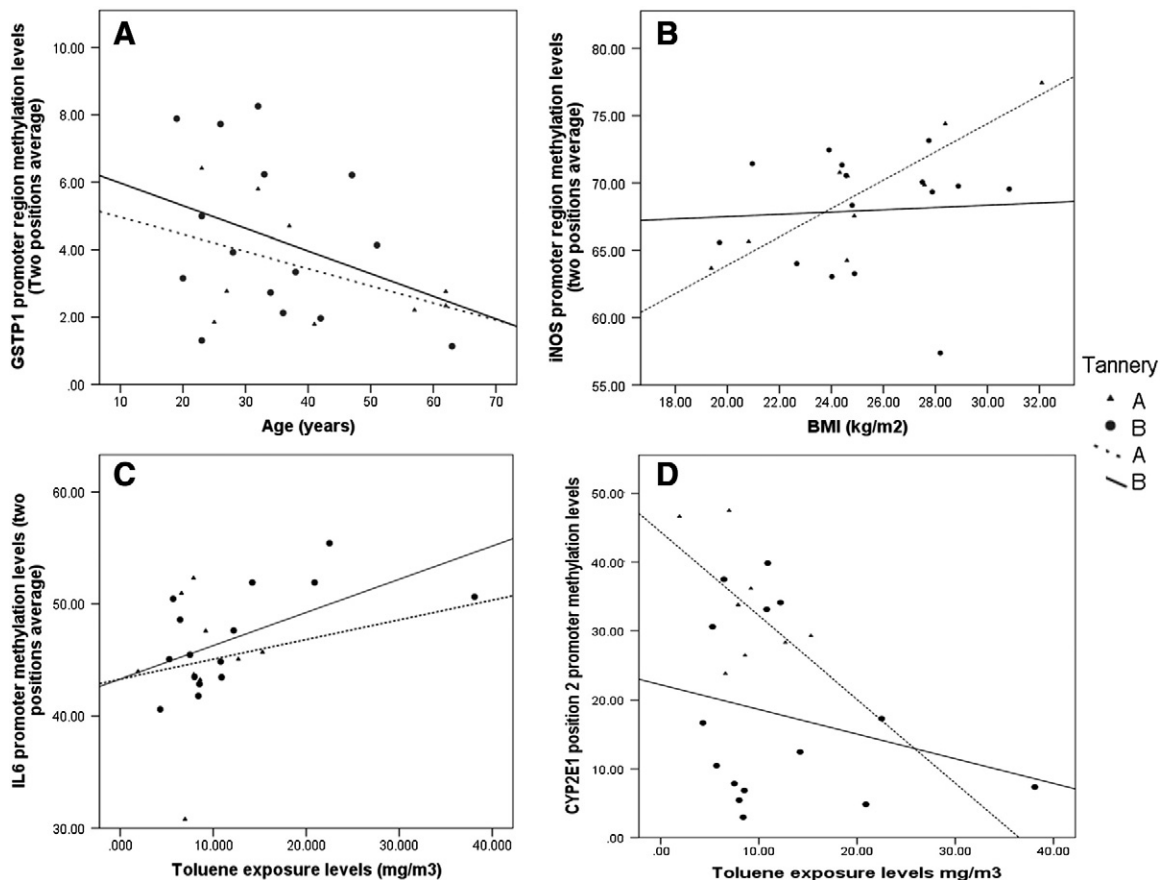
Fig. 1. Correlations between gene promoter methylation (averaged at two or more CpG sites) and study variables and covariates in exposed group. A: association of *GSTP1* promoter methylation (averaged at two CpG sites) with age *(r = −.39, p = 0.02)*. B: association of *iNOS* promoter methylation (averaged at two CpG sites) with BMI *(r = .39, p = 0.02)*. C: association of *IL6* promoter methylation (averaged at two CpG sites) with toluene exposure levels *(r = .44, p = 0.01)*. D: association of *CYP2E1* promoter methylation (position number 2) with toluene exposure levels *(r = −.36, p = 0.04)*.
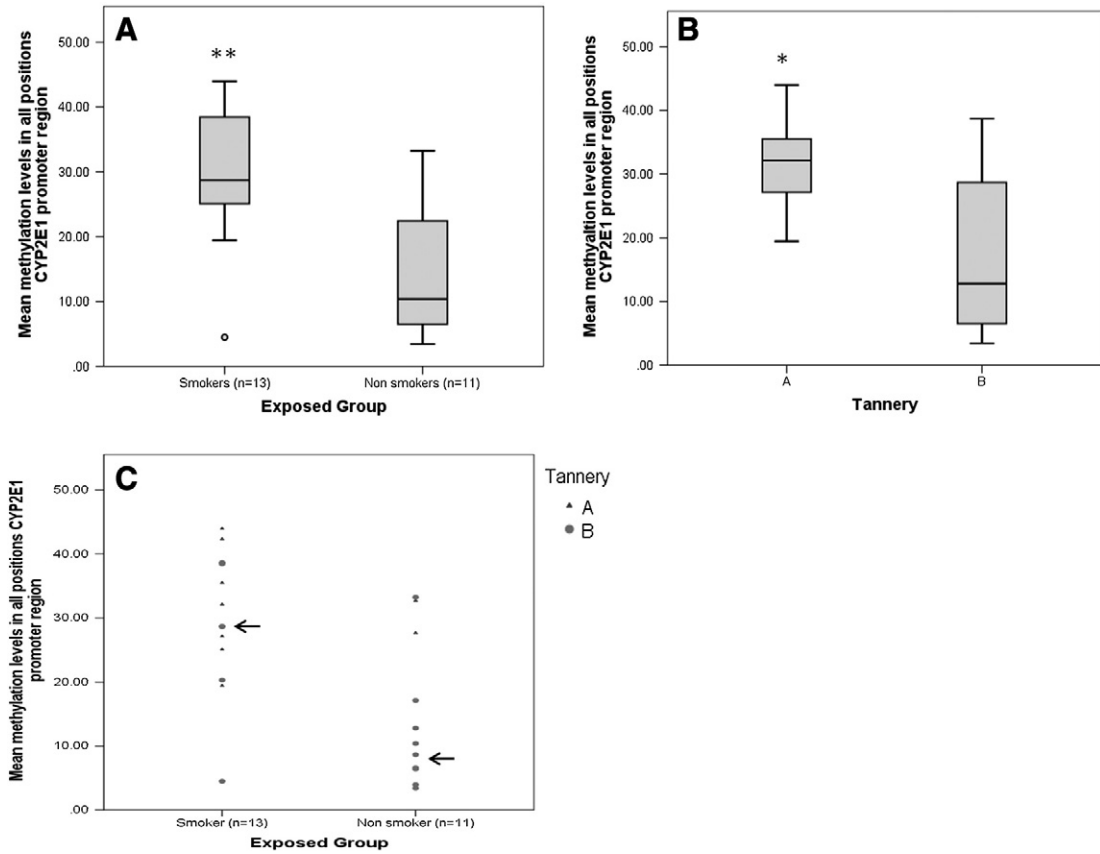
Fig. 2. A: Mean methylation levels for all the positions studied (five, averaged) at the *CYP2E1* promoter in smokers and nonsmokers from the exposed group. B: Mean methylation levels for all the positions studied (five, averaged) at the *CYP2E1* promoter in workers from tannery A and B. C: Distribution values for methylation levels in smokers identified by tanneries. **$p < 0.005$; *$p < 0.05$. the arrow is the reference for identifying the median value.

After performing the general lineal model with covariates, taking as dependent variables the *CYP2E1* mean methylation levels of the five positions studied, as well as position two in the same promoter, we corroborated that *GSTP1* position 1 as well as *SOD1* position 3 showed a significant relationship (data not shown). Also, categorical variables "smoking habit" (*CYP2E1* Mean methylation levels for all positions vs. *GSTP1* position 1) and "tannery" (*CYP2E1* position 2 vs. *SOD1* position 3) also showed a significant *p* value in all models. In Fig. 3, we have included slopes for the mentioned covariates, divided by subgroup.

Discussion

In this work, we analyzed DNA promoter methylation status in several genes involved in different cellular pathways known to be affected by toluene exposure. We compared the results obtained from two different groups: tannery workers (occupationally exposed to toluene) and administrative workers (non-exposed). In tannery workers we have found significant relationships for biological parameters such as age and *GSTP1* promoter methylation in more than one CpG (position),
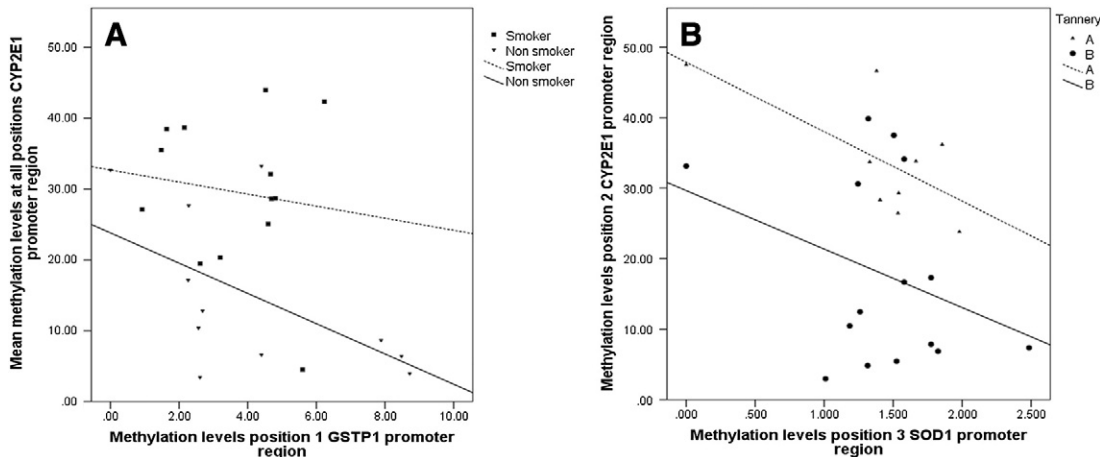
Fig. 3. A: Simple lineal regression of mean methylation values from 5 positions at the *CYP2E1* promoter vs. methylation levels in position 1 at the *GSTP1* promoter gene in the exposed group ($r = -.37$, $p = 0.03$). B: Simple lineal regression of methylation levels in position 2 of at the *CYP2E1* promoter vs. methylation levels in position 3 at the *SOD1* promoter ($r = -.34$ $p = 0.04$).

while *iNOS* promoter (two positions) methylation was affected by BMI in both groups. Exposure parameters such as individual toluene exposure levels affected at least one position in *IL6* and *CYP2E1* promoter methylation. Other personal characteristics in the exposed group such as smoking status and working in a certain tannery showed also an effect on *CYP2E1* promoter methylation. Finally, since CYP2E1 activity has been extensively related with the triggering of oxidative stress, we show an approach to demonstrate a possible crosstalk in promoter methylation status in the *CYP2E1* gene with other genes involved in the mentioned pathway. Since promoter methylation has been shown to be inversely correlated with transcription silencing and then gene expression (Gordon et al., 2014; Hackett and Surani, 2013; Jia and Salvatore, 2013; Kaut et al., 2012) we further contrast our findings with other reports showing an alteration in protein and mRNA expression, as well as DNA promoter methylation in another genes.

Toluene levels, cumulative time of exposure and promoter methylation correlations

Previous studies about global DNA methylation in human blood had already reported either positive or negative significant correlations for benzene exposure levels (Byun et al., 2013) and persistent organic pollutants (Kim et al., 2010; Rusiecki et al., 2008); while only one study found a significant correlation for specific promoter methylation (*APC* gene) and exposure levels of PM 10 and PM 2.5 (Hou et al., 2011). Our results are then in line with the mentioned reports. However, nor the genes studied here neither the effect of toluene exposure on promoter specific methylation had been investigated. The positive correlations between toluene exposure with all positions in *IL6* gene promoter (Fig. 1C) and *GSTP1* promoter promoter methylation (Table 3) found here suggest that, as toluene levels increase, leukocytes may become less responsive to combat infections (due to a possible reduction in IL6 production) and also more prone to increased reactive oxygen species (ROS) formation effects, because of a potential reduction in GSTP1 expression. While there is no information about GSTP1 expression in human toluene exposure, a study performed on mice has shown that chronic combined toluene and n-hexane exposure increased scores of bacteria colony forming units isolated from liver, lung and spleen. It also increased granulomatous areas in the same organs compared to animals carrying the same infection without such exposure (Palermo-Neto et al., 2001). These observations support the postulated effects for the IL6 reduction induced by toluene exposure in humans. The *IL6* promoter methylation increment, related with toluene exposure levels, seems to be independent of the exposed subgroups, since the same trend for a positive correlation was observed for participants either for tannery A or tannery B (Fig. 1C, slopes). Also, in spite of the fact that we identified a case as an outlier in tannery B (dot near the value of 40 mg/m³), the general linear model with covariates did not identify a significant *p* value for tannery A compared to tannery B.

Our finding showing a negative correlation between *CYP2E1* promoter methylation and toluene levels (Fig. 1D), seems to concur with the observations obtained by Mendoza-Cantú et al. (2006) who described that, as toluene exposure increases, CYP2E1 mRNA also increases in leukocytes. This observation in our subgroups (tannery A and tannery B) has the same trend (negative correlation) in both subsets of participants. Even in the subgroup with less number of participants (tannery A) the r² value for the slope is higher than in the subgroup with more participants (tannery B, values not shown, interpreted in the figure by the slope inclination). CYP2E1 expression regulation by DNA methylation in humans was first observed during development, where hypermethylation in important sequences from this enzyme gene in fetal liver was associated with null expression, as well as a demethylation in the same regions was observed as the cause of the gradual expression increase in later developmental stages in neonates (Vieira et al., 1996). Under pathological conditions, such as Parkinson Disease, decreased methylation has recently been reported in the *CYP2E1* promoter region

in brains from affected persons, with a consequent increment of CYP2E1 mRNA (Kaut et al., 2012).

Regarding cumulative time of exposure and methylation levels in peripheral leukocytes, a significant relation have been already reported by Wan et al. (2012); these authors also found a negative correlation for two methylation sites and smoking habit. The positive correlation of cumulative time of exposure to IL6 promoter methylation (Table 3) levels observed here can be interpreted in a similar way to the increased toluene exposure levels. In other words, chronic toluene exposure may cause a reduced responsiveness of peripheral leukocytes to pathogen bacteria invasion.

Age and promoter methylation correlations

In general, it is accepted that age positively correlates with DNA methylation in different human tissues (Horvath et al., 2012). However, some studies performed in blood from healthy donors have shown either no correlation in promoter methylation (Zhang et al., 2012) or a demethylation relation (Gowers et al., 2011). In human chemical exposure, Kim et al. (2010) showed a statistically significant inverse correlation between age and markers for global DNA methylation (*Alu* repeat element). Our results, showing age of the exposed group correlating positively with *SOD1* promoter methylation (Table 3) and negatively with *GSTP1* (Fig. 1A), point out to an effect on gene expression of these enzymes derived from the chronic, constant exposure to toluene combined with age increment. Both enzymes have to do with the defense system against ROS formation, a landmark of toluene exposure. In the case of SOD1, the result suggesting a silencing in this enzyme expression as age increases (due to high promoter methylation) seems contrary to the expected, since leukocytes exposed to toluene should increase the expression of any anti-ROS component. However, taking into account that age is an important factor for an imbalance in the cellular redox state, this relationship may be interpreted as an additional risk showing that, as age increases, people exposed to toluene are more prone to develop the oxidative effects caused by such exposure. The inverse relation found for *GSTP1* methylation levels and age, on the other hand, implies that an increased expression of this enzyme is needed in these people not only because of the oxidative stress increment, but also for toluene detoxification, since GSTP1 participates in phase II reaction in order to synthesize the toluene metabolites detected in urine and used for biomonitoring this compound.

As judged by the slope depicted by tannery, the same trend for a negative correlation is observed for both subgroups (Fig. 1A), making no difference for this effect between subsets of exposed participants. The meaning of this relation could also be interpreted based on the following facts: a) age is the most known risk factor for cancer (Vineis and Wild, 2014), and b) *GSTP1* promoter is hypermethylated in some cancers, although this phenomenon has been claimed recently more as a secondary effect, rather than a causal event (Pellacani et al., 2014). Then it can be proposed that the possible effect of age on *GSTP1* promoter demethylation is a precondition to develop the carcinogenic effect attributable to toluene exposure, as claimed in some epidemiological studies (Breccia et al., 2012; Cocco et al., 2010; Costantini et al., 2008; Jung et al., 2007).

Body mass index and promoter methylation correlation

It is well known that expression of the adipokine TNF-α correlates with BMI and the overweight comorbidities (Khan et al., 2013). Toluene occupational exposure has also been claimed as a deregulator of TNF-α production in white blood cells (Haro-García et al., 2012). Present results (Table 3) suggest that the obesity-dependent increase of TNF-α production in exposed people raises the risk for a chronic inflammatory state, making it a vicious circle. It is worth to mention that, contrastingly, Haro-García and co-workers found a reduction of TNF-α formation. It

has to be considered that participants in that study were exposed not only to toluene but also to benzene and xylene. Once again, as in the case of age, a possible epigenetic link between toluene exposure and cancer could be established, taking into account the well-known relation between obesity and some cancers (Khan et al., 2013) and also because the same result was not observed in the control group (Table 3), making toluene exposure an important variable for increasing TNF-α formation by leukocytes. The increment in the two CpGs of *iNOS* promoter methylation, proportional to augmented BMI in E group (Fig. 1, panel B), suggests at a first sight a decrement in this important enzyme's expression as a consequence of toluene exposure in obese participants.

However, the same correlation was found for *iNOS* position 2 in the nonexposed group (r = .4, p = 0.02, Table 3), talking about a physiological event, rather than a consequence of the occupational exposure to toluene. It is important to mention that an apparent separated effect is observed in exposed subgroups, since slope for tannery B (the subgroup with more participants showing the higher BMI levels) is almost flat ($r^2 = 0.01$) compared to tannery B ($r^2 = 0.48$). However, results from the general linear model did not show statistical differences for the categorical variable "tannery" (A or B). Also, as shown in Tables 1 and 2, no significant differences were observed in subgroups regarding age, cumulative time of exposure or toluene exposure levels, and no other covariate but BMI was found to affect the methylation of *iNOS* promoter that could explain this different trend.

Differences in CYP2E1 promoter methylation for exposed subgroups

Our results regarding *CYP2E1* promoter methylation in exposed subgroups (tannery A vs. tannery B, smokers vs. nonsmokers), showed significant differences for both subsets (Figs. 2A and B), observing higher methylation levels for tannery A, and also for the smokers' subgroup. In a first analysis, we can attribute these differences only to the fact that most workers in tannery A were current smokers (seven out of a total of nine, Table 1), however, six smokers (almost half of the total) worked at tannery B. In Fig. 2C, conclusion regarding smoking habit is the real factor responsible for the higher methylation levels becomes more evident, since all methylation values in smokers from both tanneries are above the median value (arrow mark) in nonsmokers, with the exception of one case in smokers (dot between 0 and 10 value) which is even identified as an outlier (Fig. 2A) and comes from tannery B. We speculate that this difference in *CYP2E1* promoter methylation is due to a co-exposure effect, mainly for the presence of benzene in cigarette smoke, but also to an increased toluene exposure compared to nonsmokers, since both solvents are present in tobacco smoke (Rustemeier et al., 2002). In animals coexposed either intermittently or continuously to both solvents, a hepatic CYP2E1 activity increment has been shown (Wetmore et al., 2008; Bird et al., 2010) as compared to those groups exposed to only one compound. However, no difference in hepatic CYP2E1 mRNA levels was observed in a murine model of toluene and 1 butanol coexposure (Ishidao et al., 2006). Human cultured lymphocytes, when exposed to a benzene metabolite (Zhang et al., 2011) have shown increased levels of CYP2E1 mRNA, similar to *in vivo* results in response to occupational toluene exposure (Mendoza-Cantú et al., 2006). CYP2E1 activity, determined by the chlorzoxazone assay, on the other hand, was not affected in both subsets of exposed smokers and exposed non-smokers (Jiménez-Garza et al., 2012); these difference (no change in enzymatic activity, but an evident modification in promoter methylation status) supports the idea that epigenetic modifications may be a used as very early biomarkers for detecting detrimental changes associated to toluene exposure.

Correlations between promoter methylation levels in different genes

We have found significant correlations between different promoter methylation genes (Fig. 3). The biological relation of CYP2E1 with SOD1 relies in the ROS formation caused by toluene itself, but also

in the ROS formation as a result of CYP2E1 activity. In a physiological scenario, cells would react to ROS formation by expressing components (in this case enzymes) to counteract such formation and to avoid, as far as possible, the detrimental effects of it. In line with the former statement, a ROS formation increment has been observed in animal aging, concomitant to increased expression of CYP2E1 protein and its mRNA, as well as an increased SOD1 activity but a decreased activity of CYP2E1 in hepatic tissue (Wauthier et al., 2006). However, in lymphocytes from alcohol exposed animals, similar results have been observed for CYP2E1 expression, but not for activity in leukocytes, where this last parameter was increased (Sharma et al., 2012), contrary to the aging process. In the same participants studied in this work, a reduced CYP2E1 activity had been observed (Jiménez-Garza et al., 2012), but in this case the systemic approach (chlorzoxazone metabolism) gives information about the global result of this enzyme activity in all tissues where it is expressed, namely brain, intestine, lungs, and liver. Our results do not concur with the observed effects in animals, because the methylation correlations suggest an increment in CYP2E1 expression with a simultaneous reduction in the antioxidant defense component production (SOD1). In this scenario, exposed persons have a cumulative risk, derived from the ROS production by toluene exposure itself, but also for that derived from CYP2E1 activity, observed already in leukocytes and derived from such enzymatic activity (Sharma et al., 2012).

The link between CYP2E1 and GSTP1 seems to be more logical in a physiological context, since the former is a phase I enzyme and the later a phase II enzyme, both participating in xenobiotic biotransformation. Our results can be a proof of a possible epigenetic crosstalk during drug metabolism process, indicating that, while decreased *CYP2E1* promoter methylation occurs (augmenting its expression), a correspondent increased methylation in the *GSTP1* promoter is taking place (decreasing its expression), modulating by this mechanism the time-dependent, sequential steps needed in toluene metabolism, this is: while CYP2E1 is working on the toluene molecule (adding a functional group in order to decrease its biological activity, or phase I) there is no need of a GSTP1 expression and vice versa (Fig. 3, panel A). Under the scenario already proposed, where GSTP1 (a phase II enzyme) expression is secondary to CYP2E1 activity (a phase I enzyme), and SOD1 expression is precluded by CYP2E1 activation due to oxidative stress triggering, the findings about a different contribution in the possible epigenetic crosstalk from categorical variables ("smoker" and "tannery") could be explained as follows: i) Being a smoker is a factor causing a delay in GSTP1 activation; this can be concluded analyzing the r^2 value of the slope for smokers (0.01) compared to nonsmokers (0.3; slope, Fig. 3A). In other words: in people exposed to toluene who do not smoke, *GSTP1* promoter methylation diminishes (augmenting then this enzyme's expression) with a more linear relationship relative to *CYP2E1* promoter methylation, behaving in the sequential way it has to be. In smokers, on the other hand, this crosstalk becomes less linear, so GSTP1 expression may be reduced or retarded, affecting in this way the efficacy for toluene metabolism and its posterior metabolite excretion. The underlying mechanism could be CYP2E1 active site saturation due to the simultaneous metabolism of toluene plus other chemicals present in tobacco smoke. ii) In workers from tannery B ($r^2 = 0.1$ vs. 0.45 from tannery A), behavior of *SOD1* methylation resembles that of *GSTP1* in exposed smokers, that is, this sequential SOD1 activation, precluded by CYP2E1 activity, is reduced in those workers. Explanation of this reduction could rely on toluene exposure level differences: in Table 2 we can see that, although not significant, toluene exposure levels in tannery B were higher compared to tannery A. A plausible explanation is that most of the working days, toluene levels are higher in tannery B compared to tannery A. We only sampled one day, and in spite of sampling was performed in the same day in both tanneries (in order to avoid seasonal variance due to temperature and humidity), we can speculate that all the time, toluene exposure levels are higher in tannery B. Another fact that supports this assumption is the amount

of production (leather) which is higher in tannery B. The underlying mechanism could be then a reduction in CYP2E1 activity due to higher toluene exposure levels.

Limitations of the study

Since results were obtained from whole blood DNA samples, they reflect the epigenetic effects on all kinds of white blood cells. It is not possible to know if toluene exposure epigenetically affects white blood cell subpopulations in the same way.

Conclusion

DNA *de novo* methylation, a biological modification in major part caused by environmental exposure may be a suitable tool for detecting several cellular modifications that could trigger the development of future ailments in people chronically exposed to toluene. This epigenetic modification can also be the key for understanding how other interacting factors such as age, obesity, cumulative time of exposure and smoking habit may accelerate the sub cellular detrimental effects attributed to oxidative stress, a toxic cellular mechanism closely related to CYP2E1 activity. Implications of these results have to do not only with occupational exposure but also with environmental exposure, since this compound is present at considerable amounts in urban areas, having increased concentrations indoors compared to outdoors.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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