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Early hematological and immunological alterations in gasoline station attendants exposed to benzene

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ABSTRACT

Introduction: Elucidation of effective biomarkers may provide tools for the early detection of biological alterations caused by benzene exposure and may contribute to the reduction of occupational diseases. This study aimed to assess early alterations on hematological and immunological systems of workers exposed to benzene.
Methods: Sixty gasoline station attendants (GSA group) and 28 control subjects were evaluated. Environmental and biological monitoring of benzene exposure was performed in blood and urine. The potential effect biomarkers evaluated were δ-aminolevulinate dehydratase (ALA-D) activity, CD80 and CD86 expression in lymphocytes and monocytes, and serum interleukin-8 (IL-8). The influence of confounding factors and toluene co-exposure were considered.
Results: Although exposures were below ACGIH (American Conference of Governmental Industrial Hygienists) limits, reduced ALA-D activity, decreased CD80 and CD86 expression in monocytes and increased IL-8 levels were found in the GSA group compared to the control subjects. Furthermore, according to multiple linear regression analysis, benzene exposure was associated to a decrease in CD80 and CD86 expression in monocytes.
Conclusions: These findings suggest, for the first time, a potential effect of benzene exposure on ALA-D activity, CD80 and CD86 expression, IL-8 levels, which could be suggested as potential markers for the early detection of benzene-induced alterations.

1. Introduction

Gasoline is a complex mixture of saturated and unsaturated hydrocarbons and is composed mainly of paraffins, olefins and aromatics (Keenan et al., 2010; Keretetse et al., 2008). Aromatic compounds are predominantly benzene, toluene and xylene (BTX) (De Palma et al., 2012; Hopf et al., 2012; Keretetse et al., 2008). Among these constituents, benzene stands out for its hazardous effects on human health (Keretetse et al., 2008; Rekhadevi et al., 2010; Tunsaringkarn et al., 2011). Airborne benzene vapors generated from gasoline are accompanied by high concentrations of many other hydrocarbons, which share very similar metabolism and detoxification pathways. Simultaneous exposure to these other hydrocarbons may have a significant impact on benzene toxicity (Keenan et al., 2010).

Benzene has been classified as a Group I carcinogenic chemical by the International Agency for Research on Cancer (IARC, 2012), and it is known to cause the hematologic disorders leukemia and myelodysplastic syndrome and lead to several deleterious effects on many other biological systems after long-term exposures (De Palma et al., 2012; Uzma et al., 2010). The hematopoietic system is

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highly sensitive to the toxic effects of benzene. Exposure to benzene is associated with bone marrow depression and aplastic anemia, resulting in a progressive decrease in erythrocytes, thrombocytes and each of the various types of leukocytes (Sun et al., 2009; Weisel, 2010; Wong et al., 2010). Consequently, benzene exposure induces immune system depression, which increases the risk for opportunistic infections and compromised immune surveillance (Tunsaringkarn et al., 2011; Uzma et al., 2010).

Biological monitoring has been used as a potential tool for better assessing integrated benzene exposures and may contribute to the diagnosis and treatment of occupational diseases (Hays et al., 2012). Biomarkers of effect are a heterogeneous group that can reflect early biological changes in response to benzene toxicity, indicative of health risks (Uzma et al., 2010). Simultaneous evaluation of biomarkers of exposure and effect may be useful in the estimation and reduction of risks caused by occupational exposure to benzene. Such assessments may also be adopted for preventative health initiatives by acting effectively on health surveillance for improving the safety of occupationally exposed workers (De Palma et al., 2012; Tunsaringkarn et al., 2011).

The present study aimed to assess potential biomarkers to detect early alterations on hematological and immunological systems of gasoline station attendants (GSA), a recognized professional category exposed to risks of benzene. For this purpose, environmental and biological monitoring was performed. The toxic effects of benzene on blood cells were assessed through usual hematological parameters and by a supposed novel biomarker of hematological alteration, the enzyme δ-aminolevulinate dehydratase (ALA-D), which is involved in the heme group biosynthesis from hemoglobin. The benzene-induced impairment in immune system was estimated by the expression of co-stimulatory molecules of the T-cell activation (CD80 and CD86) and serum interleukin-8 (IL-8), proposed as important effect biomarkers involved in the bone marrow toxicity induced by benzene. Additionally, we analyzed the possible influence of additional hydrocarbons present in gasoline, as well as other confounding factors such as age, smoking and exposure time.

2. Materials and methods

2.1. Study population

A total of 88 male subjects participated in this study. The exposed group consisted of 60 gasoline station attendants (GSA) from Rio Grande do Sul, Brazil. GSA were divided into two groups: non-smokers ($n=44$) and smokers ($n=16$). All subjects had been working in their current job position for at least 6 months. The control group consisted of 28 subjects who were non-smokers and who had no history of occupational exposure to benzene.

A previous sample size calculation was performed in order to ensure that the number of subjects evaluated in this work was enough to observe reliable statistical differences. It was accomplished based on a previous study of our research group with a population of workers exposed to toluene. The results of sample size calculation showed that at least 24 controls and 48 exposed subjects should be included in the work to detect statistical difference with 80% power and a significance level of 0.05.

Each subject answered a structured questionnaire about general health, lifestyle, smoking status and history of exposure. Individuals with chronic diseases such as diabetes, hypertension, cardiovascular disease, hypo-or hyperthyroidism, and pathologies related to hepatic and renal function were not included in the study.

Personal monitors were used to assess the BTX concentration in the breathing zone during the daily work shifts (approximately 8 h). After air sampling, the samples were capped, transported to the lab and stored at −20 °C until analysis.

Urine and blood samples were collected from all participants at the end of the work shift following 3–4 consecutive days of exposure. Urine samples (50 mL) were collected for BTX metabolites and creatinine determination. The samples were stored in polyethylene bottles at −80 °C until further analysis. Two EDTA vacuum blood collection tube were collected. The first one was used for BTX quantification, after collection, it was immediately sealed and kept at −80 °C until further analysis. Another EDTA vacuum blood collection tube was used for hemogram parameters determination. A blood-heparin tube was collected for the determination of ALA-D activity and adhesion molecules expression (CD80 and CD86). An aliquot (0.5 mL) of whole blood with heparin was stored into eppendorf tubes and kept at −80 °C until ALA-D enzyme analysis. The quantification of CD80 and CD86 expression was performed in the remainder whole blood with heparin, which was refrigerated at 4 °C and processed within 24 h. Another vacuum blood collection tube without anticoagulant was collected and centrifuged at 1500*g* for 10 min at room temperature. The obtained serum was stored at −80 °C until the measurement IL-8 levels.

This study was approved by the Ethics Committee in Research from the Federal University of Rio Grande do Sul/RS (No. 21728/11) and informed consent was obtained from each participant.

2.2. Exposure assessment

Personal passive samplers (SKC 575-002®) were used to evaluated airborne BTX (benzene, toluene, *o-*, *m-*, *p*-xylene) exposure. The content of BTX presented in each sampler was desorbed with dichloromethane and analyzed by gas chromatography and flame ionization detection (GC-FID; PerkinElmer, USA). GC column Innowax (25 m, 0.2 mm, 0.4 μm) was used. The initial oven temperature was 40 °C, and the temperature was increased by 4 °C min^{-1} until it reached 53 °C, followed by an oven ramp rate of 40 °C min^{-1} up to 200 °C. The FID detector temperature was kept at 250 °C. The limits of detection (LOD) and quantification (LOQ) were 0.5 and 1.7 μg mL^{-1} to benzene, respectively; 0.3 and 1.0 μg mL^{-1} to toluene, respectively; 0.3 and 1.2 μg mL^{-1} to *o*-xylene, respectively; 0.3 and 1.1 μg mL^{-1} to *m*-xylene, respectively; and 0.4 and 1.3 μg mL^{-1} to p-xylene, respectively.

The concentration of lead (Pb) in gasoline was determined by ICP-MS (PerkinElmer-Sciex, USA), according to the method previous described by Mateus et al. (2013), with LOD and LOQ of 0.01 μg L^{-1} and 6.0 μg kg^{-1}, respectively.

Blood BTX levels were analyzed by headspace GC-FID (PerkinElmer, USA). For analysis, 1 mL of blood was added to 4 mL of NaCl 30% (w/v) and 10 μg mL^{-1} nitrobenzene (internal standard, IS; 100 μL) into 20 mL headspace vials. After vortexing (10 s), the samples were incubated at 60 °C for 15 min and then injected into the GC-FID system. A GC OV-1 column (30 m, 0.32 mm, 1 μm) was used. The initial oven temperature of 80 °C was maintained for 2 min, increased by 10 °C min^{-1} to 110 °C, and then increased by 20 °C min^{-1} until the temperature reached 200 °C, which was maintained for 3 min. The FID detector temperature was kept at 250 °C. The LOD and LOQ were 1.0 and 3.0 ng mL^{-1} to all the organic solvents analyzed, respectively.

Quantification of a urinary metabolite of benzene (trans,trans-muconic acid-t,t-MA) was carried out by high-performance liquid chromatography with UV detection (HPLC-UV; Shimadzu, USA) after solid phase extraction (SPE) according to a previously described analytical method (Ducos et al., 1990), with modifications (SPE cartridge: 100 mg; sample volume: 200 μL). Quantification of urinary metabolites of toluene (hippuric acid-HA) and xylene (methylhippuric acid-mHA) was simultaneously performed by HPLC-UV (Bulcão et al., 2008). The creatinine concentration was

Table 1
Main characteristics of the study population.

	Control group ($n=28$)	GSA group ($n=60$)	
		Non-smokers ($n=44$)	Smokers ($n=16$)
Age (years)	30.4 ± 1.8	33.9 ± 1.25	
		33.8 ± 1.4	34.3 ± 2.6
Time of occupational exposure (months)	NA	119.8 ± 12.8	
		114.0 ± 13.5	135.8 ± 30.8
Alcohol eventual drinkers [n (%)]		49 (81.7)	
	21 (75.0)	35 (79.5)	14 (87.5)
Continous medication use [n (%)]		12 (20.0)	
	0 (0.0)	8 (50.0)	4 (25.0)

NA: not applicable.
The values are expressed as mean ± SEM.
[n (%)]: total number found per group and in parenthesis the perceptual.

measured by spectrophotometry as previously described (Jaffé, 1886) using commercial kits (Doles reagents, Brazil). According to the methods used to quantify urinary BTX metabolites, the LOD and LOQ were 0.05 and 0.1 μg L^{-1} to t,t-MA, respectively; 0.001 and 0.003 g g^{-1} for creatinine to HA, respectively; and 0.003 and 0.008 g g^{-1} for creatinine to mHA, respectively.

2.3. Hematological biomarkers

The erythrograms, white blood cell analyses and platelet analyses were performed using a hematology analyzer (Cobas Micros 60 System, USA).

δ-aminolevulinate dehydratase (ALA-D) activity was assayed in erythrocyte lysates according to a previously described method (Sassa, 1982). The enzymatic activity was also determined in the presence of the reducing agent dithiothreitol (DTT).

2.4. Immune system biomarkers

CD80 and CD86 expression by lymphocytes and monocytes was analyzed by flow cytometry. The samples were processed within 24 h. Erythrocytes lysis was performed using an ammonium chloride solution (0.13 M) and the leukocytes were resuspended with PBS buffer. 10^6 leukocytes were incubated with PE-conjugated anti-CD80 and FITC-conjugated anti-CD86. The antibodies were diluted 1:100 with PBS and incubated in the dark at 4 °C for 20 min. Cells were analyzed by FACSC Canto II Flow Cytometer (Becton Dickinson, San Jose, CA) with FlowJo Software (TreeStar). Lymphocyte and monocyte cells were identified by manual gating according to side scatter and size.

Serum interleukin-8 (IL-8) levels were quantified by BD™ cytometric bead array (CBA) human inflammation cytokines kit (BD Biosciences, USA) according to the manufacturer's instructions.

2.5. Statistical analysis

Data analysis was performed with IBM SPSS Statistics software (version 19). All study variables were tested for normality using the Shapiro–Wilk test. Comparisons between the groups were carried out using the Student's *t*-test and Mann–Whitney *U*-test. When necessary, an ANOVA and Kruskal–Wallis test were employed to compare groups, followed by multiple comparison tests (Tukey's post hoc test). The results were expressed as mean ± standard error of the mean (SEM) or median (interquartile range), according to the distribution of the variables. Spearman's rank correlation tests were used to evaluate the relationship between variables. Multiple linear regression models were applied to verify the involvement of benzene exposure on the biomarkers of effect. The influence of confounding factors (age, smoking habits and exposure time) and toluene co-exposure were considered. Variables that had a non-normal distribution were log transformed to be included in multivariate regressions. The significance level for all tests was $p \leq 0.05$.

3. Results

The main characteristics of the study population assessed by the questionnaire are summarized in Table 1.

Airborne BTX exposure is reported in Table 2. The GSA group had higher levels of exposure to BTX than the control group ($p < 0.001$). While median personal BTX exposure levels seemed to be slightly higher in GSA smokers compared to non-smokers, the differences were not statistically significant ($p > 0.05$). However,

Table 2
Airborne BTX exposure and urinary metabolites.

	Control group ($n=28$)	GSA group ($n=60$)	
		Non-smokers ($n=44$)	Smokers ($n=16$)
Benzene (μg m^{-3})	42.0 (34.1–52.4)	144.2 (58.1–2207.5)[a]	165.5 (102.7–2207.5)[b]
		70.7 (58.1–1067.1)[b]	
Toluene (μg m^{-3})	107.2 (78.4–172.3)	345.3 (213.0–3945.1)[a]	430.3 (219.7–3945.1)[b]
		328.0 (213.0–945.1)[b]	
o-Xylene (μg m^{-3})	30.0 (21.6–65.3)	182.0 (29.8–627.4)[a]	347.4 (29.8–627.4)[b]
		154.4 (30.5–313.5)[b]	
m-Xylene (μg m^{-3})	20.0 (17.4–25.5)	30.3 (23.4–58.3)[a]	32.3 (23.4–32.8)[b]
		29.8 (24.0–58.3)[b]	
p-Xylene (μg m^{-3})	21.2 (18.4–27.0)	29.7 (21.0–36.5)[a]	31.0 (21.0–34.6)[b]
		28.8 (22.3–36.5)[b]	
t,t-MA (μg g^{-1} creatinine)	70.0 (50.0–120.0)	334.4 (190.0–600.0)[a]	400.0 (300.0–600.0)[b]
		320.0 (190.0–450.0)[b]	
HA (g g^{-1} creatinine)	0.3 (0.2–0.4)	0.3 (0.2–0.7)	0.3 (0.3–0.6)
		0.3 (0.2–0.7)	
m-HA (g g^{-1} creatinine)	n.d	n.d	n.d
		n.d	

t,t-MA: trans,trans-muconic acid; HA: hippuric acid; mHA: methylhippuric acid; n.d.: not detectable. m-HA (LOD): 0.003 g g^{-1} creatinine.
The values are expressed as median (interquartile range).
[a] $p < 0.001$ compared to the control group (Mann–Whitney *U*-test).
[b] $p < 0.001$ compared to the control group (Kruskal–Wallis test/Tukey test).

data showed that all values found to be below the ACGIH (American Conference of Governmental Industrial Hygienists) limits.

In relation to the content of lead in the gasoline, no detectable levels of this metal were found in the analyzed samples (LOD = 0.01 μg L^{-1}).

Regarding blood biomarkers of exposure to BTX, only 10 (22.7%) non-smoker GSA and 8 (50%) smoker GSA presented detectable levels of blood benzene (B-BZ) (LOD = 1.0 ng mL^{-1}). The median B-BZ concentration in GSA smokers and GSA non-smokers were 7.1 ng mL^{-1} (3.7–9.9 ng mL^{-1}) and 5.5 ng mL^{-1} (3.0–12.6 ng mL^{-1}), respectively, and no statistical significant difference was observed. Similar levels of blood toluene (B-TOL) were found in both GSA groups, with median concentrations of 8.7 ng mL^{-1} (6.7–9.7 ng mL^{-1}) and 8.5 ng mL^{-1} (6.9–9.8 ng mL^{-1}) for GSA smokers and non-smokers, respectively. The control group did not present measurable levels of B-BZ and B-TOL (LOD = 1.0 ng mL^{-1}). Blood xylene levels were undetectable in all study subjects (LOD = 1.0 ng mL^{-1}).

Higher urinary t,t-MA levels were measured in the GSA group compared to that in the controls ($p < 0.001$; Table 2). In the same way, higher urinary t,t-MA content was found in GSA smokers compared to GSA non-smokers, however no significant difference was observed within the GSA groups. Although t,t-MA levels were higher in GSA group compared to the control group, the median values were below the biological exposure index (BEI: 500 μg g^{-1} creatinine) established by ACGIH.

The urinary biomarker of exposure to toluene was not significantly different among the groups ($p > 0.05$; Table 2). HA median values were below the biological limit value for occupationally exposed people (BEI: 1.6 g g^{-1} creatinine; ACGIH). Furthermore, the urinary biomarker of exposure to xylene could not be detected in any members of the study population (LOD = 0.003 g g^{-1} creatinine; Table 2).

Since we did not find significant differences in the exposure assessment, between both GSA groups (smokers and non-smokers), the analysis of effect biomarkers was performed without taking into account smoking habits.

The results of classical hematological parameters are summarized in Table 3. Decreased red blood cells and hemoglobin content were observed in GSA group relative to the control group ($p < 0.001$). In addition, increased neutrophil counts were observed in GSA group when compared to the control group ($p < 0.001$). Other hematological parameters did not present significant differences between the groups. All hematological parameters are within the reference values for adult men.

Fig. 1 shows ALA-D enzymatic activity results, indicating that GSA group showed reduced ALA-D activity in relation to the control group ($p < 0.001$). When the reducing agent DTT was added, increase of 28.0% and 16.0% in ALA-D activity were obtained for the GSA group and the control group, respectively, corresponding to the ALA-D reactivation index (%). ALA-D/DTT activity for GSA group remained lower than the control group ($p < 0.001$; Fig. 1), and significant difference were observed in ALA-D activity when assayed either with or without DTT in both groups ($p < 0.001$).

Table 3
Hematological parameters.

	Control group ($n=28$)	GSA group ($n=60$)
Red blood cells (10^6 mm^{-3})	5.2 ± 0.1	4.9 ± 0.1[a]
Hemoglobin (g dL^{-1})	15.1 ± 0.1	14.6 ± 0.1[a]
Hematocrit (%)	44.2 ± 0.3	43.6 ± 0.4
White blood cells (10^3 mm^{-3})	7.1 ± 0.3	7.3 ± 0.2
Lymphocytes (%)	36.2 ± 1.3	36.3 ± 1.0
Monocytes (%)	8.9 ± 0.4	8.6 ± 0.4
Neutrophils (%)	52.0 ± 1.5	57.4 ± 0.9[a]
Eosinophils (%)	2.3 ± 0.2	2.1 ± 0.2
Basophils (%)	0.5 ± 0.1	0.4 ± 0.1
Platelets (10^3 mm^{-3})	242.7 ± 9.6	235.2 ± 7.7

The values are expressed as mean ± SEM.

[a] $p < 0.001$ compared to the control group (Mann–Whitney *U*-test).

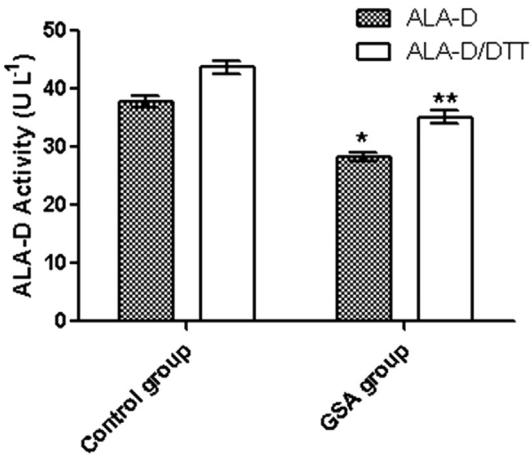

Fig. 1. ALA-D enzymatic activity assayed with or without the reducing agent dithiothreitol (DTT). The data are represented as mean ± SEM. *$p < 0.001$ compared to the control group, **$p < 0.001$ compared to the control group (Mann–Whitney *U*-test).

Expression of CD80 and CD86 in lymphocytes and monocytes are shown in Fig. 2. Although no significant difference was found in CD80 expression in lymphocytes between the studied groups ($p > 0.05$; Fig. 2A), CD86 expression in lymphocytes was lower in the GSA group compared to the control group ($p < 0.05$; Fig. 2A). Monocyte CD80 and CD86 expression was significantly lower in the GSA group in relation to the control group ($p < 0.05$; Fig. 2B).

Serum IL-8 levels were higher in the GSA group (4.0 ± 1.0 pg mL^{-1}) compared to the control group (2.0 ± 0.1 pg mL^{-1}; $p < 0.001$).

Spearman's correlations were analyzed in all subjects, as a single sample. Airborne benzene and toluene exposure were well correlated ($r = 0.83$; $p < 0.001$). No significant correlations were found between airborne exposure to benzene or toluene and their blood levels, respectively ($p > 0.05$ for both). A significant correlation was observed between airborne benzene exposure and urinary t,t-MA levels ($r = 0.35$; $p < 0.01$). However, no significant correlation was observed between air toluene concentration and urinary HA levels ($p > 0.05$).

Airborne exposure to benzene and toluene levels, as well as urinary t,t-MA concentrations, were significantly correlated to ALA-D activity, expression of CD80 and CD86 in monocytes and IL-8 levels (Table 4). No significant correlation was found between expression of CD80 and CD86 in lymphocytes and airborne exposure to benzene or toluene and urinary t,t-MA concentrations ($p > 0.05$).

Additionally, exposure time was also correlated to ALA-D activity ($r = -0.67$; $p < 0.001$), expression of CD80 and CD86 in monocytes ($r = -0.32$; $p < 0.01$ and $r = -0.56$; $p < 0.001$, respectively) and IL-8 levels ($r = 0.40$; $p < 0.001$).

Table 5 displays the main results of the multiple linear regression models. In relation to ALA-D, the best-fit model revealed that exposure time was the only factor that significantly contributed to reduced enzymatic activity. Urinary t,t-MA was modeled as the best predictor for decreased CD80 and CD86 expression in monocytes. Individual toluene exposure, age and cigarette smoking did not show significant influences on the multiple regression models.

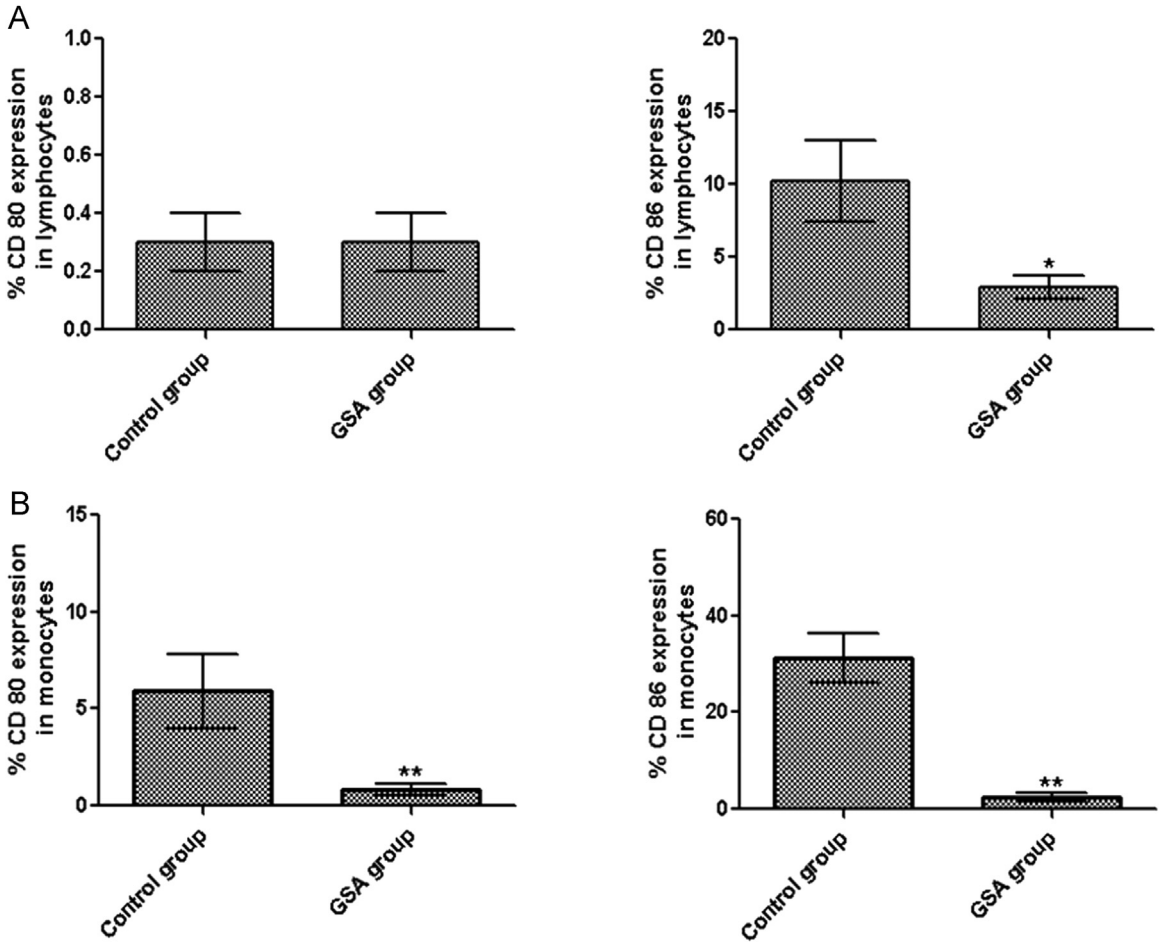

Fig. 2. (A) Expressions of CD80 and CD86 in lymphocytes. (B) Expressions of CD80 and CD86 in monocytes. Values are expressed as mean ± SEM. $^{*}p < 0.05$ compared to the control group, $^{**}p < 0.001$ compared to the control group (Mann–Whitney *U*-test).

4. Discussion

Characterization of human exposure to pollutants is an important element in occupational studies, risk evaluations and risk management (Weisel, 2010). Environmental and biological monitoring are complementary tools used to assess integrated exposures, occupational health risk evaluation and occupational health practice (Manno et al., 2010).

In the present study, environmental monitoring indicated that gasoline station attendants were exposed to levels of BTX well below the threshold limit value time-weighted average proposed by ACGIH to the working environment (ACGIH, 2014). Furthermore, our data were also lower than those reported in Indian and Thai studies, when both the personal BTX exposure or benzene levels alone are considered (Chanvaivit et al., 2007; Rekhadevi et al., 2010; Ruchirawat et al., 2010; Uzma et al., 2010). The concentration of benzene exposure observed in our study may be due to the reduced percentage of this organic solvent in gasoline (EPA, 2011). Nevertheless, even reduced levels of benzene exposure may represent a critical factor in the development of leukemia. This is because benzene cannot be completely eliminated from gasoline, and there are no safe limits for exposures to carcinogens, such as benzene (Carrieri et al., 2006; Weisel, 2010). The World Health Organization (WHO) has estimated that the risk of developing leukemia is approximately six cases per million among people who experience lifetime exposure to benzene concentrations of 1 $\mu g\ m^{-3}$ in the air (WHO, 1996). Following this line, a previous study of our group showed that reduced levels of benzene exposure could be linked to genotoxicity, which if not be repaired may contribute to the carcinogenicity development (Moro et al., 2013). Based on these data, occupational exposure to benzene should be considered as a serious public health concern (Manini et al., 2008).

Despite reduced exposure levels, airborne benzene and toluene exposure were highly correlated with each other, indicating the co-exposure occurrence in gasoline stations.

Table 4
Spearman's correlations between airborne benzene and toluene exposure and t,t-MA *versus* biomarkers of effect ($n=88$).

	Airborne benzene exposure ($\mu g\ m^{-3}$)	Airborne toluene exposure ($\mu g\ m^{-3}$)	t,t-MA ($\mu g\ g^{-1}$ creatinine)
ALA-D ($U\ L^{-1}$)	$r=-0.40$ ($p<0.001$)	$r=-0.31$ ($p<0.01$)	$r=-0.40$ ($p<0.001$)
CD80+ in monocytes (%)	$r=-0.37$ ($p<0.01$)	$r=-0.40$ ($p<0.001$)	$r=-0.36$ ($p<0.01$)
CD86+ in monocytes (%)	$r=-0.48$ ($p<0.001$)	$r=-0.53$ ($p<0.001$)	$r=-0.41$ ($p<0.001$)
IL-8 ($pg\ mL^{-1}$)	$r=0.42$ ($p<0.01$)	$r=0.39$ ($p<0.01$)	$r=0.37$ ($p<0.01$)

t,t-MA: trans,trans-muconic acid; ALA-D: δ-aminolevulinate dehydratase; IL-8: interleukin 8.

Table 5
Multiple linear regression models.

	ALA-D (U L^{-1}) R^2= 0.351		Log CD80+ in monocytes (%) R^2= 0.288		Log CD86+ in monocytes (%) R^2= 0.566	
	β	p-Values	β	p-Values	β	p-Values
Log airborne benzene exposure (μg m^{-3})[a]	0.064	0.734	−0.224	0.261	−0.244	0.119
Log airborne toluene exposure (μg m^{-3})[a]	−0.194	0.313	−0.211	0.296	−0.140	0.378
Log t,t-MA (μg g^{-1} creatinine)[a]	−0.112	0.329	−0.278	0.044	−0.379	< 0.001
Age (years)[a]	0.295	0.143	−0.043	0.846	−0.093	0.575
Exposure time (months)[a]	0.752	0.001	0.081	0.730	−0.197	0.262
Smoking[b]	0.046	0.713	0.047	0.749	−0.109	0.313

ALA-D: δ-aminolevulinate dehydratase; t,t-MA: trans,trans-muconic acid.

[a] Categorical variable.
[b] Continuous variable.

Our data showed significant differences in t,t-MA levels between GSA group and the control group. Additionally, t,t-MA excretion correlated with airborne benzene exposure, which confirmed the relevance of this biomarker, even at reduced levels. HA, in contrast, showed no significant differences between the studied groups. This indicated a low specificity of this biomarker in the monitoring of low-level toluene exposures, which has already been evidenced in our previous studies with painters (Moro et al., 2010, Moro et al., 2012). The validity of hippuric acid as a biomarker of occupational exposure to toluene has often been questioned, since occupational exposures to toluene have been gradually decreasing and also because urinary hippuric acid can also be found in nonexposed subjects, originated from foods, drinks and drugs containing benzoic acid or benzoates, thereby compromising the use of this metabolite in the biomonitoring of low-level toluene exposures (Roma-Torres et al., 2006; Truchon et al., 1999; Ukai et al., 2007).

The hematotoxic effects caused by chronic benzene exposure have been thoroughly studied; thus, classical hematological parameters are routinely evaluated in such cases (Kirkeleit et al., 2008; Robert Schnatter et al., 2010). Our results showed that benzene occupational exposure affected red blood cell, hematocrit and neutrophil counts; however, these parameters remained within the reference values. According to previously described work, alterations in the number of blood cells are not especially sensitive indicators of early benzene effects (Swaen et al., 2010). These data have been reinforced by experimental studies showing that low levels of hydroquinone exposure, an immunotoxic metabolite of benzene, does not alter bone marrow or peripheral leukocyte and erythrocyte counts but does affect the function of circulating leukocytes (Ribeiro et al., 2011; Shimada et al., 2012).

Our study showed that circulating mononuclear cells from gasoline station attendants, especially monocytes, presented a marked reduction on expression of co-stimulatory molecules CD80 and CD86. According to multiple linear regression analysis, t,t-MA was the only factor that significantly contributed to this reduction, demonstrating the involvement of benzene in the reduced expression of these immunological accessory molecules. CD80 and CD86 molecules are expressed on antigen-presenting cells, where they play a key role in the activation of T-cells, as well as in the production of cytokines associated with adaptive immune responses (Chanan-Khan et al., 2011; Dolen and Esendagli, 2013; Mohamed et al., 2012; Pardoll, 2012). The lack of both surface molecules may lead to hyporesponsiveness or to the development of anergic T cells (Dai et al., 2009).

The lymphocyte population studied here are B-cells, which are professional antigen-presenting cells in which both CD80 and CD86 have specific functions. For example, CD80 expression is a marker for different subsets of B-memory cells (Tomayko et al., 2010), CD86 signaling can synergize with Toll like receptor 2 (TLR2) signaling to activate resting B cells independent of B-cell receptor signaling (Jain et al., 2013), and both molecules are involved in B-cell malignancies (Greaves and Gribben, 2013).

Studies have reported that leukemia patients express lower levels of CD80 and CD86 compared to healthy people (Dai et al., 2009; Zhao et al., 2012). In addition, the abnormal expression of CD80 and CD86 is considered one of the major mechanisms of tumor cell escape from immune surveillance, which may be one of the pathogenic mechanisms of some types of leukemia (Costello et al., 1998; Dai et al., 2009). Based on the fact that occupational exposure to benzene is closely linked to the development of leukemia, the assessment of these co-stimulatory molecules in benzene-exposed workers may serve as an interesting tool in biological monitoring.

Furthermore, we observed increased IL-8 levels in the GSA group when compared to the control group. Interleukin-8 is a well established cytokine involved in tumor progression acting on tumor growth and survival (Toutirais et al., 2003). The elevated IL-8 levels in gasoline station attendants could be related to the involvement of polyphenolics metabolites of benzene in inducing bone marrow toxicity (Bironaite et al., 2004). The induction of apoptosis in progenitor cells in bone marrow by benzene metabolites may play an important role in induction of aplastic anemia by benzene. Moreover, excessive apoptosis in bone marrow has been suggested to be a likely cause of myelodysplasia, which is considered a pre-leukemic disorder and has also been associated with benzene exposure (Bironaite et al., 2004). Increased IL-8 expression correlates well with the increased neutrophil counts observed since both are indicative of an enhanced innate immunity.

We also assessed ALA-D activity in an attempt to evaluate early biomarkers of hematological alterations caused by benzene exposure. The enzymatic activity of this enzyme was lower in the GSA group compared to the control group. ALA-D inhibition compromises the heme synthesis of hemoglobin, which may ultimately contribute to the development of anemia (Baierle et al., 2010), a common hematotoxic effect resulting from benzene exposure (Robert Schnatter et al., 2010; Smith, 2010). Furthermore, this enzymatic inhibition leads to ALA (5-aminolevulinic acid) accumulation. ALA behaves as an endogenous pro-oxidant, increasing the formation of reactive oxygen species (ROS) in cells (Muzyka et al., 1998; Muzyka et al., 2004). The involvement of ROS in the inhibition of ALA-D was evaluated by using the reducing agent DTT, a thiol-reducing agent that has been used *in vitro* to prevent and/or reverse ALA-D inhibition induced by oxidizing agents (Valentini et al., 2007). Although ALA-D activity, when assayed with DTT, was increased in GSA group, it was still significantly lower than the activity observed in the control group,

suggesting that mechanisms, other than oxidation of thiol groups, could be involved in ALA-D inhibition.

Linear correlations showed that the decreased ALA-D activity was correlated with higher t,t-MA excretion, along with airborne benzene and toluene exposures. These findings suggested the possible involvement of benzene and toluene on ALA-D inhibition. Undetectable levels of lead in gasoline also contributed to this hypothesis. A previous study from our group indicated toluene as the major inducer of ALA-D inhibition in painters exposed to a mixture of organic solvents (Moro et al., 2010). To the best of our knowledge, the correlation between benzene exposure and ALAD inhibition has not been previously shown, as its action had already been proposed on other enzymes responsible for the heme biosynthesis (Muzyka et al., 2004). According to multivariate analysis, exposure time was the only factor which significantly contributed to the reduction of enzymatic activity. This suggests that chronic exposure to benzene may contribute to ALA-D inhibition, which, in the long-term, can supposedly cooperate in the decrease of enzymatic synthesis. However, further studies are needed to completely elucidate the involvement of this enzyme in the development of anemia in subjects exposed to benzene

In addition, exposure time was correlated with the biomarkers of alterations observed (enzyme ALA-D, CD80 and CD86 expression in monocytes, IL-8 levels). This suggests that chronic benzene exposure is associated with the progress of various alterations that in the long-term can result in damage, which substantiates the reliability of these biomolecules as suitable biomarkers of effect.

Our study had some limitations. The lower number of GSA smokers in relation to non-smokers may have contributed to the lack of influence of smoking habits on the biomarkers evaluated. Also, the functional effects of changes in CD80 and CD86 expression could not be assessed in the present study. Additional studies are necessary to elucidate the involvement of benzene on the regulatory mechanisms of CD80 and CD86 and to determine whether these molecules could be predictors of alterations that contribute to leukemia development in workers chronically exposed to benzene, and whether such changes could impair the activation of adaptive immunity in response to pathogens.

5. Conclusions

In the present study, we suggested, for the first time, potential early biomarkers of hematological and immunological alterations (activity ALA-D inhibition, CD80 and CD86 inhibition expressions in monocytes and increase of IL-8 levels) for the biological monitoring of benzene exposure. The data indicate that benzene exposure may be correlated with enhanced activity of the innate immune system, while adaptive immunity may be compromised as indicated by reduced expression of the co-stimulatory molecules CD80 and CD86. Taken together, these findings may contribute to the early detection of benzene-induced alterations of the immune system after occupational exposures and also may promote the development of preventive health measures for exposed workers, ultimately improving their quality of life.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Approval ethics committee for human research

All study was conducted in accordance with national and institutional guidelines for the protection of human subjects. This study was approved by the Ethics Committee in Research from the Federal University of Rio Grande do Sul/RS (No. 21728/11).

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