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RESEARCH ARTICLE

Quantitative measurement of oxidative damage in erythrocytes as indicator in benzene intoxications

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ABSTRACT

The metabolism of aromatic hydrocarbons by the organism forms products that cause cell death depending on the type of exposure. Benzene exposure has been linked to oxidative stress, hepatic damage, aplastic anemia, and hematopoietic cancer as lymphoid and myeloid leukemia. However, there are not fast methods to evaluate chronic benzene exposure in human blood. The objective of this work was the evaluation of the correlation between oxidative damage with benzene exposure and the level of cellular plasma membrane stability (CPMS) in erythrocytes to use it as a future indicator to determine the grade of benzene intoxications. CPMS *in vitro* assays were used to evaluate damage for benzene, toluene, and xylene. Erythrocytes CPMS assays *in vitro* shows a progressive reduction with benzene, toluene, and xylene suggesting that aromatic hydrocarbons complexity favors CPMS damage. Eight groups of Wistar rats ($n = 5$) were used to study the level of damage on CPMS by acute and chronic benzene administration. Enzymatic, metabolic, histological, and oxidative damage tests were performed. Acute administration (100 μL/100 g/single dose) showed a decrease of 66.7% in CPMS, while 63.6% for chronic administration (5 μL/100 g/every 2 days/3 months) showing a correlation with liver damage principally (transaminases activity increase, glycogen level decrease, and high oxidative damage). Tissue damage was observed in bone marrow, kidney, spleen, and lungs. Benzene produces damage on CPMS depending on the exposure time and dose. The CPMS technique could be used as an important aromatic hydrocarbons intoxication indicator.

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Introduction

Nowadays there are several disease conditions related to oxidative stress (OxS) conducing to constant production of reactive free radicals (FR). These compounds bind to structural and functional biomolecules changing their conformation and biological function (Martindale and Holbrook 2002; Lin and Beal 2006; Monaghan et al. 2009). The FR are products with increased metabolic activity in the organism that can be caused by various mechanisms as metabolism of compounds like drugs, food, ultraviolet radiation, physical activity, protein mutations, and the exposure to certain pollutant compounds in the environment pathways (Fisher-Wellman and Bloomer 2009). We usually can get antioxidant compounds through daily diet to help our body to fight against OxS fulfilling a function of 'free radical scavengers', avoiding in conjunction with endogenous antioxidants cellular oxidative damage (Rizzo et al. 2010; White et al. 2014).

When cells remain exposed to oxidant agents causes damage dependent on the stress exposure time. The most serious damage to cells is the alteration of genetic information caused by mutations in DNA and/or activation of the cell death pathway like apoptosis and necrosis (Buttke and Sandstrom 1994; Monaghan et al. 2009). Endogenous and exogenous antioxidant compounds are important to maintain homeostasis between oxidant and reducing agents (Tsao and Deng 2004; Mancuso et al. 2012).

Lipid peroxidation involves the reactions between FR (mainly reactive oxygen species or ROS) and structural lipid from plasmatic membrane and membrane organelles. Additionally, ROS may also react with proteins and carbohydrates forming 'adducts' or oxidation products as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Nieto et al. 2002; Zarkovic 2003; Carini et al. 2004; Long et al. 2006).

Plasma membrane has several functions like high selectivity filter, intracellular/extracellular ion concentrations control by a diverse set of ion channels (voltage-gated, ligand, ATP, etc.) in order to maintain optimum electrolyte homeostasis carrying out the processes and reactions necessary for a healthy body. Increase in FR generates a chain reaction with the membrane components and destabilizes intracellular/extracellular balance disrupting cell function activating

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apoptosis. This process is dependent on the grade of stability of plasma membrane (Chasis and Mohandas 1986).

Polycyclic aromatic hydrocarbon compounds are metabolized in the liver and bone marrow producing highly toxic secondary metabolites. High exposure to OxS by benzene in the cells may develop cancer like acute and chronic lymphoid and myeloid leukemia, solid tumors, hepatic damage, hematological alterations, aplastic anemia, central nervous depression, skin irritation, and immunotoxicity (Green et al. 1981; Velasco Lezama et al. 2001; Khalade et al. 2010). Benzene is widely used in industry and is in the 20 top chemicals of higher production volume, besides it is an important environmental pollutant that has been awarded together with polycyclic aromatic compounds as a product with toxic effects (Snyder and Hedli 1996; Ross 2000; Snyder 2000; Ikenaka et al. 2013). Little is known about benzene metabolism, however it is well-known the epoxidation by CYP450-2E1 as the first transformation in a couple of unstable species like benzene oxide and oxepin (Snyder 2007).

The mechanisms of bone marrow toxicity occur as follows: (1) inhibition of mitosis, (2) inhibition of interleukin-1 synthesis, (3) covalent binding to proteins and nucleic acids, (4) inhibition of DNA polymerase, (5) chromosomes damage, and (6) FR formation as ROS. Cellular functions are altered by the change in the chemical environment that causes OxS (Laskin et al. 2000; Snyder 2004; Ibrahim et al. 2014). Cell environment damage causes membrane plasmatic permeability triggering cell death that could be observed and measured in tissues, enzymatic alteration and unbalance of oxidizing and reducing endogenous compounds.

In Mexico, the main causes of death respect to chronic degenerative disease are cardiovascular diseases, diabetes mellitus, and malignant tumors (INEGI 2011; SINAIS 2010). These results are similar to global data reported by World Health Organization (WHO) in 2012. Over time chronic degenerative disease has been linked to OxS and FR.

The purpose of this study was to analyze the relationship between the grade of cellular plasma membrane stability (CPMS) to different benzene concentrations and the grade of damage found in major organs where it is metabolized in order to analyze the toxic action by OxS and its relation to various etiologies and development of diseases that in our times increase day by day.

Methods

Animals and experimental protocols

Male Wistar rats ($n = 40$) weighing 200 ± 10 g were randomly assigned to eight groups of five rats each: two control groups and six benzene dosing groups. Animals were treated in a stable environment with controlled temperature ($22 \pm 2\,^{\circ}C$), humidity, and light (12 hours light: dark cycle). All the experiments reported here obey to university regulations on the care and handling of experimental animals (NOM-062-ZOO-1999) and to environment care (NOM-087-ECOL-SSA1-2002) with consent and approval of Bioethics Committee and Investigation of the Autonomous University of Zacatecas from 17 August 2015 to 1 August 2017 (UAZ-2015-36853). Benzene administration was done intraperitoneally. For the acute model, we used three different doses per group (5, 50, and 100 μL/kg, single dose). In the chronic model, we administered 5 μL/100 g every 48 hours for 1, 2, and 3 months. The control groups were administrated with mineral oil (ANALYTYKA, Monterrey, NL, Mexico) to compare the results of the damage models.

At the end of the experimental models, animals were weighted and sacrificed under anesthesia in ether atmosphere to obtain total blood and serum by cardiac puncture. Different organs such as liver, kidney, bone marrow, spleen, brain, and lung were removed to analyze lipid peroxidation grade and histological indicators.

ALT and AST activity

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by AST and ALT BioSystems kit. About 100 μL of serum samples were added to 1 mL of sample reagent and incubated at 30 °C (Multi-Blok-Heater Melrose Park, IL, USA). After 1 minute we took the first absorbance value and then three more readings were taken every minute (Beckman DU-65, Fullerton, CA, USA). Finally, the increment value per minute was obtained (ΔH/min). These values were multiplied by the factor 1745 to obtain a value in units of U/L.

Measure of glycogen concentration

The Fong method (Fong et al. 1953) was used to analyze liver glycogen concentration, which is based on the hydrolysis of glycogen to glucose units by potassium hydroxide in order to react with anthrone in sulfuric acid forming a green colored complex that is measured spectrophotometrically. One gram of liver was hydrolyzed with 3 mL of 30% KOH and boiled for 30 minutes. Then, we carefully dilute 1:250. Then 2 mL of anthrone (200 mg/dL in concentrated sulfuric acid) were added to 1 mL of this mixture in cold-water bath (Felmont, Monterrey, NL, Mexico). To the controls we added 1 mL of water and 2 mL of anthrone. Samples were placed in a boiling water bath for 10 minutes. The tubes were cooled on ice bath for 5 minutes. Samples were read in a spectrophotometer (Perkin-Elmer JUNIOR Model 35, Maywood, IL, USA) at 620 nm against control sample. Dextrose calibration curve (Monterrey, NL, Mexico) was used to quantify glucose unites in the sample. Factor conversion was used to convert glucose to glycogen units.

Lipid peroxidation

Damage of tissues by OxS induces the formation of oxidation products as MDA. This lipid peroxidation product was analyzed by thiobarbituric acid reactive substances (TBARS) measurement described by Mihara and Uchiyama (1978). The method is described briefly, 10% liver homogenate (Wheaton, Millville, NJ) in 1.15% KCl was prepared from animals livers, eliminating the remains of connective tissue through double gauze. An aliquot of 0.5 mL was added into

test tube adding 3 mL of 1% phosphoric acid (J T Baker, Mexico) and 0.6% thiobarbituric acid (J T Baker, Phillipsburg, NJ). The tubes were placed in boiling bath for 45 minutes and then cooled for 5 minutes in ice water bath to add 3 mL 1-butanol (HYCEL, Zapopan, Jalisco, Mexico). Tubes were mixed and centrifuged for 5 minutes at 2500 rpm (C-600, SULBAT, Mexico) to separate in two phases, collecting the butanol phase and reading absorbance at 535 nm. 1, 1, 3, 3-Tetramethoxypropane calibration curve (Aldrich, MO) was used to evaluate MDA concentration.

CPMS

The method of Navarro was used to analyze CPMS (Navarro et al. 1993). The technique is described briefly; 1 mL of animal blood was acquired from cardiac puncture and mixed gently with 1 mL Alsever reagent (0.05% citric acid (ANALYTYKA, Monterrey, NL, Mexico), 0.8% sodium citrate (Monterrey, NL, Mexico), 0.42% sodium chloride (KEM, León, Gto, Mexico) and 2% dextrose). Subsequently it was centrifuged at 2000 rpm for 5 minutes and the supernatant was removed. Sterile saline was added, mixed, and centrifuged at 2000 rpm for 5 minutes. This procedure was repeated 4–6 times until a crystalline supernatant was obtained (zero hemolysis). Finally, a cell suspension was prepared at 10% v/v. A mixture with 1 mL of phosphate buffer (0.15 M pH 7.4), 2 mL of distilled water and 0.5 mL of 10% suspension of erythrocytes (3.5 mL total volume) was made. For total hemolysis control, 0.5 mL of 10% erythrocytes suspension and 3 mL of distilled water were added. Treatments and control samples were incubated at 50 °C for 30 minutes (Felisa, Zapopan, Jalisco, Mexico) and then centrifuged at 2500 rpm for 5 minutes. The content of hemoglobin in the supernatant was measured spectrophotometrically at 560 nm. CPMS percentage was calculated using the following equation:

$$\%\ \text{CPMS} = 100 - [(\text{O.D. Treatment Sample} / \text{O.D. Total Hemolysis Control}) \times 100].$$

CPMS in vitro

One healthy rat was sacrificed to isolate erythrocytes and evaluate CPMS *in vitro* by the modified method of Navarro et al. (1993). Erythrocytes were exposed to aromatic hydrocarbons like benzene, toluene, and xylene. We used different volumes of aromatic hydrocarbons (1, 5, 10, 25, and 50 µL) to evaluate CPMS in erythrocytes. Each volume of aromatic hydrocarbons was added directly on the mixture (1 mL of phosphate buffer and 2 mL of distilled water) before adding slowly 0.5 mL of 10% erythrocytes suspension over organic phase. Subsequently the mixtures were incubated at 50 °C for 30 minutes and then centrifuged at 2500 rpm for 5 minutes. We used two control samples. (1) Total hemolysis control (0.5 mL of 10% erythrocytes suspension and 3 mL of distilled water) and (2) hemolysis control in hypotonic medium (0.5 mL of 10% erythrocytes suspension, 1 mL of phosphate buffer (0.15 M, pH 7.4), and 2 mL of distilled water). The content of hemoglobin in the supernatant was measured spectrophotometrically at 560 nm. CPMS percentage was calculated using the following equation:

$$\%\ \text{CPMS} = 100 - \{[(\text{O.D. Problem Sample} - \text{O.D. hemolysis control in hypotonic medium}) / \text{O.D. Total Hemolysis Control}] \times 100\}.$$

Histological indicator

Hematoxylin eosin (HE) and Masson trichrome staining (MTS) was used to identify cell damage with respect to the administration of benzene. A Bencosme method (Bencosme 1954) was used to analyze liver, kidney, spleen, brain, pancreas, skeletal muscle, bone marrow, lungs, and heart tissues with a Carl ZEISS Axioskop 40 fluorescence microscope.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and are analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison. A discriminant classification analysis was made to observe the variability in the data obtained with respect to the measured indicators. We used STATGRAPHICS Centurion XV software (Plains, VA, USA) for Windows. Differences were considered statistically significant at $p < 0.05$.

Results

Benzene administration increases ALT and AST activity

It is well known that ALT and AST are markers of cell damage therefore we analyzed them in response to benzene administration. ALT enzyme activity in the acute model showed a gradual increase 10.8%, 26.9%, and 58.9% in animals administrated with benzene compared with the control group. In the chronic model, a gradual increase was observed in the enzymatic activity of 24.7%, 49.8%, and 60.9%. Data showed an increase directly proportional to the exposure time as shown in Table 1 and Figure 1.

Additionally, AST activity in the acute model showed a gradual increase of 31.3%, 93.4%, and 148.9% compared with control. The chronic model showed an increase directly proportional to exposure time of 46.0%, 48.5%, and 96.2% compared with control group. Individual variability of each data was

Table 1. Enzymatic and metabolic indicators.

	Doses	ALT (U/L)	AST (U/L)	Glycogen (µg glycogen per g of liver)
Acute model	Control	29.76 ± 3.25	78.26 ± 5.15	6.78 ± 0.60
	5 µL/100g	32.98 ± 2.82	102.78 ± 33.39	2.84 ± 0.73*
	50 µL/100g	37.77 ± 3.04	151.37 ± 51.82*	4.04 ± 1.16*
	100 µL/100g	47.28 ± 12.47*	194.82 ± 43.28*	1.20 ± 0.59*
Chronic model	Control	29.22 ± 8.19	79.92 ± 7.93	7.27 ± 1.25
	5 µL/100g/1 month	36.47 ± 3.68	116.65 ± 11.22*	4.77 ± 0.33*
	5 µL/100g/2 months	43.79 ± 2.01*	118.66 ± 19.15*	3.57 ± 1.05*
	5 µL/100g/3 months	47.02 ± 3.19*	156.78 ± 27.30*	3.89 ± 0.86*

The results show the value ± SD ($n = 5$).
*Statistically significant compared with the corresponding control group ($p < 0.05$).

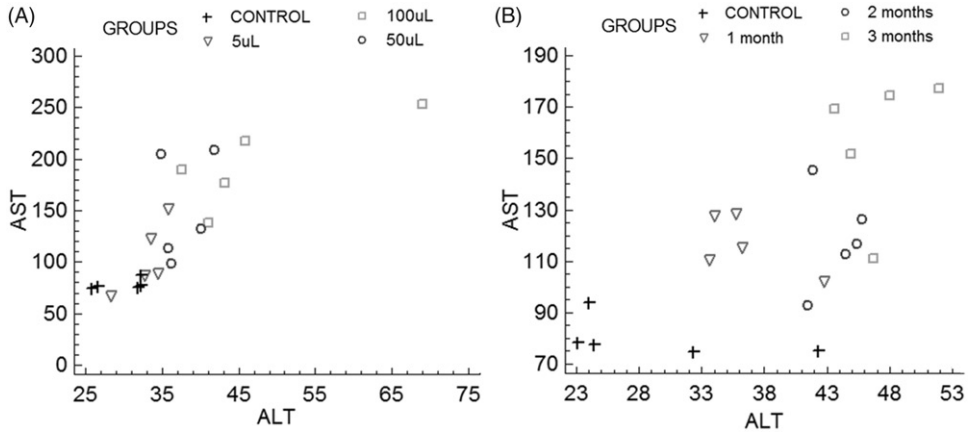

Figure 1. Acute and chronic benzene administration increases ALT and AST activity. (A) Rats were administrated with different benzene single doses (5, 50, and 100 μL per each 100 g of weight). After 24 hours the animals were sacrificed and serum samples were obtained, AST and ALT enzymatic activity was determined. Scatter diagram using a discriminant analysis shows the individual variability of each data in AST and ALT level in the acute experimental groups. Control group shows a low activity of aminotransferases compared with the administration of benzene acute doses. (B) Rats were administrated with 5 μL of benzene every two days for 1, 2, and 3 months. Animals were sacrificed after the corresponding time and serum samples were obtained and transaminases activity was determinated. A scatter diagram using a discriminant analysis shows the individual variability of each data in AST and ALT level. Aminotransferase activity was compared with benzene chronic administration.

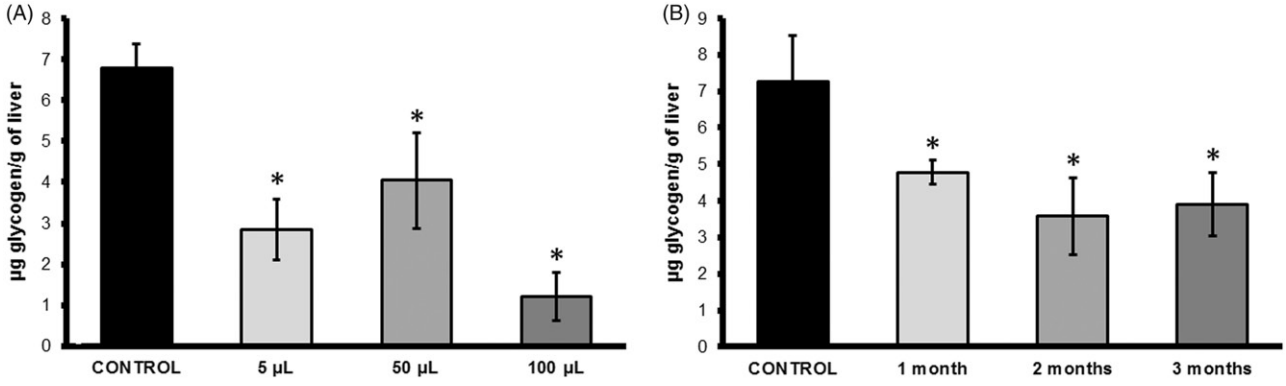

Figure 2. Exposure to benzene decreases glycogen concentration in liver. (A) Rats were administered with 5, 50, and 100 μL of benzene per 100 g of weight in a single dose. After 24 hours the animals were sacrificed and liver samples were obtained and glycogen concentration in liver was determinated. The graph shows a decrease in liver glycogen concentration dependent of acute dose (58.0%, 40.4%, and 82.1%). (B) Rats were treated with 5 μL of benzene for 1, 2, and 3 months and sacrificed after the corresponding time and liver samples were obtained and glycogen concentration per gram of liver was determinate. The graph shows a decrease dependent on dose administrated (34.0%, 50.9%, and 46.5%). We used ANOVA to analyze statistic differences indicated by * ($n = 5$, $^*p < 0.05$).

evaluated in acute and chronic benzene administration with a discriminant correlation analysis (Table 1 and Figure 1(A)). An increase in aminotransferases activity levels was observed with benzene versus control group. A similar fashion was recorded with chronic benzene administration (Figure 1(B)).

Benzene exposure decreases glycogen concentration in liver

Glycogen concentration changes could be used to evaluate hepatic cytotoxicity. As we were able to show glycogen decreases gradually in groups of benzene acute administration (58.0%, 40.4%, and 82.1%) (Table 1 and Figure 2). In the chronic model, glycogen decreased with respect to time of benzene exposure. The groups showed a decrease in the deposit of this polysaccharide of 34.0%, 50.9%, and 46.5%.

Benzene induces histological changes

We were able to show that ALT and AST activity and glycogen concentration in serum and liver, respectively, vary importantly with benzene administration. Furthermore, we observed liver, kidney, spleen, brain, pancreas, skeletal muscle, bone marrow, lungs, and heart to seek for histological alterations. No significant structural changes were observed with the administration of benzene in the acute model. However, as it can be seen from Figure 3, in the chronic model, morphological changes were evident especially in the liver and kidney. Structures of normal liver lobule were observed in control group (Figure 3(A,B)) while a loss of normal liver architecture was observed in benzene-treated rats. The presence of patchy cell death and a large amount of collagen (fibrosis) from hepatocytes cords and central veins were observed in animals treated (Figure 3(C,D)). In bone marrow, cell abnormal morphology was observed in treated rats (Figure 3(G,H)) versus controls (Figure 3(E,F)) as well as widespread necrosis and cell growth decrease (Figure 3(G,H)). Moreover, the kidney shows a high grade of necrosis and inflammation in tubules and glomerulus evidencing cell disruption and loss of normal structure (Figure 3(J–L)) compared with the control group (Figure 3(I)). In the case of spleen, moderate inflammation was observed compared with

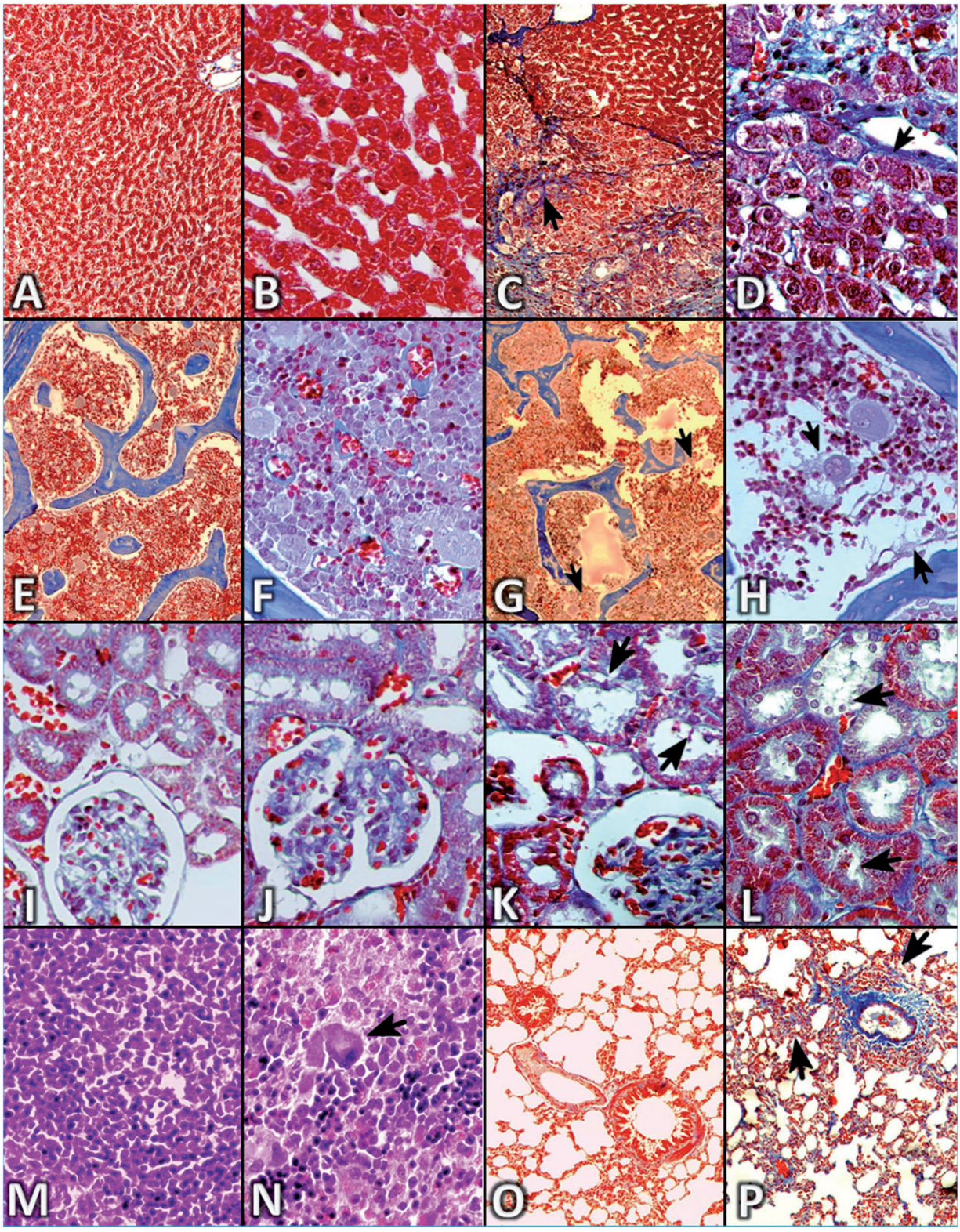

Figure 3. Benzene intoxication affects liver, bone marrow, spleen, kidney, and lungs. In the chronic model rats were treated with 5 μL/100 g of benzene for 1, 2, and 3 months. Animals were sacrificed and tissues were analyzed histologically. Pictures A (10×, MTS) and B (40×, MTS) show normal hepatocytes structure, evidencing lobules and hepatocytes defined architecture. Pictures C (10×, MTS) and D (40×, MTS) evidence that benzene exposure induces damage in liver, showing high concentration of collagen between hepatocytes (fibrotic liver) and total loss of hepatic architecture. Additionally, the presence of cell death and a large amount of collagen from cords centrilobular hepatocytes and veins were observed. Pictures E (10× MTS) and F (40× MTS) show a control group with normal structure of bone marrow, while pictures G (10×, MTS) and H (100×, MTS) show damage in bone marrow induced with benzene treatment. Immature cells decrease, cell lysis, and loss of normal bone marrow architecture were observed with chronic exposition with benzene. Picture I (40×, MTS) shows a normal kidney structure with very well defined glomerulus and tubules structures, while pictures J, K, and L (40×, MTS) show that benzene administration produces loss of normal structure of renal tubules, showing cell death in renal tubules, glomerulonephritis, fibrosis, and cell congestion. Picture M (40×, H&E) shows the spleen with normal sinusoids structures and in picture N (40×, H&E) shows a swollen spleen tissue, evidencing presence of megakaryocytes, normally these cells do not grow on this site, unless there is damage in bone marrow. Additionally multinucleated cells were observed. Picture O (40×, MTS) shows normal lung structure evidencing very well defined normal alveolar tissue, while in picture P, alveolar inflammation and fibrosis were observed.

control group (Figure 3(M)). Besides multinucleated cells and megakaryocyte cells could be identified despite they should not be in this site (Figure 3(N)). Moreover, fibrosis and alveolar inflammation was observed in lungs (Figure 3(P)), showing a significant pathological change compared with the control group (Figure 3(O)). The organs more affected with benzene were liver, bone marrow, kidney, spleen, and lungs. Cellular damage could be achieved by several changes in cell metabolism therefore we sought for lipid peroxidation (MDA).

Grade of lipid peroxidation with benzene exposition

To correlate the grade of tissue damage and enzymatic indicator with OxS, we analyzed MDA concentration in liver because it is the main metabolizing organ, kidney is the site of metabolites elimination, spleen is the organ with high blood supply and brain is related with neuronal damage because of its high lipid content. As it can be seen from Table 2, all organs show an increased MDA formation with respect to acute and chronic administration indicating possibly a high reaction between FR and metabolites derived from metabolism of benzene with biomolecules. The results show damage dependent on dose and exposure time. Liver was the organ that presented greater damage.

Benzene decreases CPMS

The indicators of toxicity evaluated here, clearly show that benzene induces OxS and cell death; therefore it is important to have a method to evaluate the exposure and the damage caused by this compound. To achieve this objective we applied the CPMS method in serum and as it was shown that the acute model shows a CPMS decrease directly related to the benzene dose administered. The CPMS in acute control group was 94.7%, which was decreased as benzene dose was increased; the percentages were 90.1%, 76.7%, 69.8% (Figure 4(A)). The chronic model showed a decrease in CPMS percentage related to time of exposure to benzene. As shown in Figure 4(B), the stability of membrane in chronic control group was from 93.0% and it consequently decreased with benzene dose administration as follows 80.5%, 66.6%, and 63.6%.

Aromatic hydrocarbons decrease CPMS in vitro *test*

In order to compare the results obtained in acute and chronic models we analyzed CPMS *in vitro*. Erythrocytes were treated with different benzene volumes (Figure 5). Furthermore, other aromatic hydrocarbons as toluene and xylene were used to evaluate if they had similar effect as benzene in erythrocytes. Benzene treatment showed a decrease in CPMS directly proportional to concentration administrated, however, showed less damage compared with toluene and xylene, showing a statistically significant difference between all amounts of substances administrated, except in the groups where 1 μL of solvent was administrated. Benzene shows significant damage over erythrocytes showing a CPMS value of 74.8% with the highest amount (50 μL) administrated in *in vitro* test. On the other hand, toluene and xylene evidenced CPMS of 59.0% and 36.6% with 50 μL, respectively. It seems the aromatic hydrocarbons chemical structural complexity increase CPMS damage.

Table 2. Lipid peroxidation levels in liver, kidney, spleen, and brain.

	DOSES	LIVER	KIDNEY	SPLEEN	BRAIN
Control	No benzene	5.08 ± 0.62	6.28 ± 0.78	6.14 ± 1.36	38.7 ± 1.95
Acute model	5 μL/100g	9.87 ± 3.11	7.61 ± 1.73	6.55 ± 0.60	43.1 ± 5.86
	50 μL/100g	7.21 ± 1.81	7.69 ± 1.90	7.04 ± 1.07	53.7 ± 13.9
	100 μL/100g	11.3 ± 1.28*	9.50 ± 2.48	9.64 ± 0.88*	49.1 ± 5.56
Chronic model	5 μL/100g/1 month	13.4 ± 2.72	15.7 ± 3.77	8.07 ± 0.27	53.7 ± 5.56
	5 μL/100g/2 months	20.6 ± 9.39*	20.6 ± 9.39	9.66 ± 0.48	53.3 ± 4.00
	5 μL/100g/3 months	23.6 ± 4.59*	21.4 ± 2.81	19.3 ± 3.39*	51.8 ± 7.32

The results show its value (μmol MDA/L) ± SD ($n = 3 \pm 1$).
*Statistically significant compared with control group ($p < 0.05$).

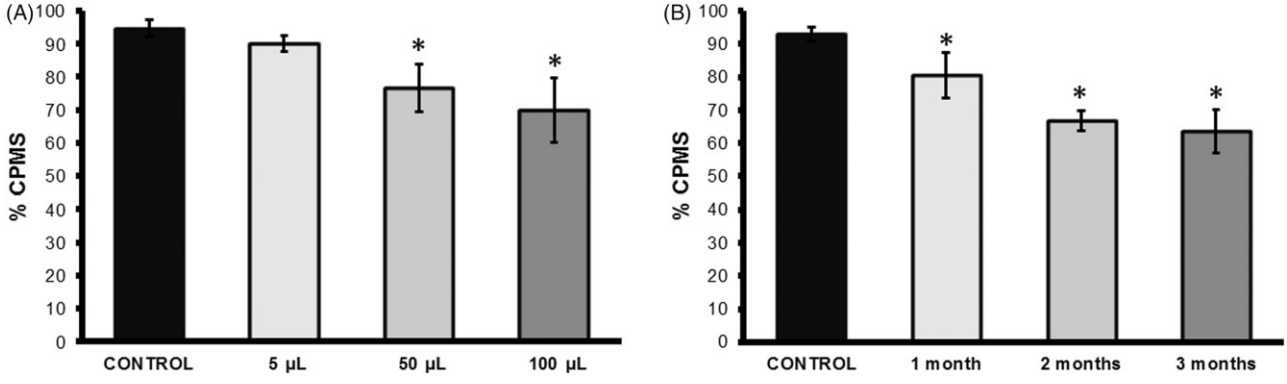

Figure 4. Benzene administration diminishes CPMS in acute and chronic models. (A) Rats were administrated with different benzene single doses (5, 50, and 100 μL per 100 g of weight). After 24 hours the animals were sacrificed and total blood sample was obtained to analyze CPMS. The graph shows a decrease in CPMS dependent on benzene acute dose (90.1%, 76.7%, 69.8%). (B) Rats were treated with 5 μL of benzene for 1, 2, and 3 months. Animals were sacrificed after the corresponding time and total blood sample was obtained, to analyze CPMS. The graph shows a decrease dependent of dose administrated (80.5%, 66.6%, and 63.6%). We used ANOVA to analyze statistic differences indicated by * ($n = 5$, *$p < 0.05$).

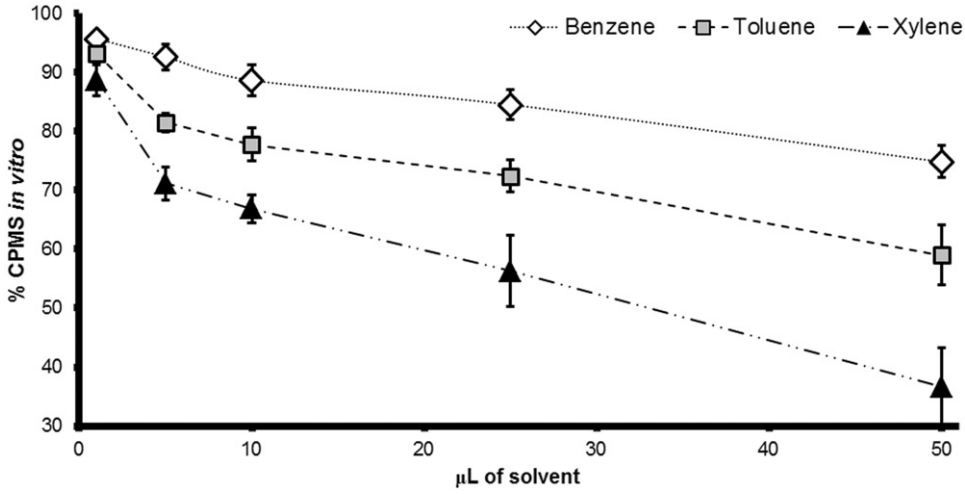

Figure 5. Benzene decreases CPMS *in vitro* test. One healthy rat was sacrificed to get isolated erythrocytes to evaluate CPMS *in vitro* adding different concentrations of aromatic hydrocarbons, including benzene, toluene, and xylene. All aromatic hydrocarbons evidenced a CPMS decrease dependent of concentration added to hypotonic medium. Benzene was the compound that caused less damage to erythrocytes compared with toluene and xylene. We used ANOVA to analyze statistic differences indicated by * ($n = 5$, $^*p < 0.05$).

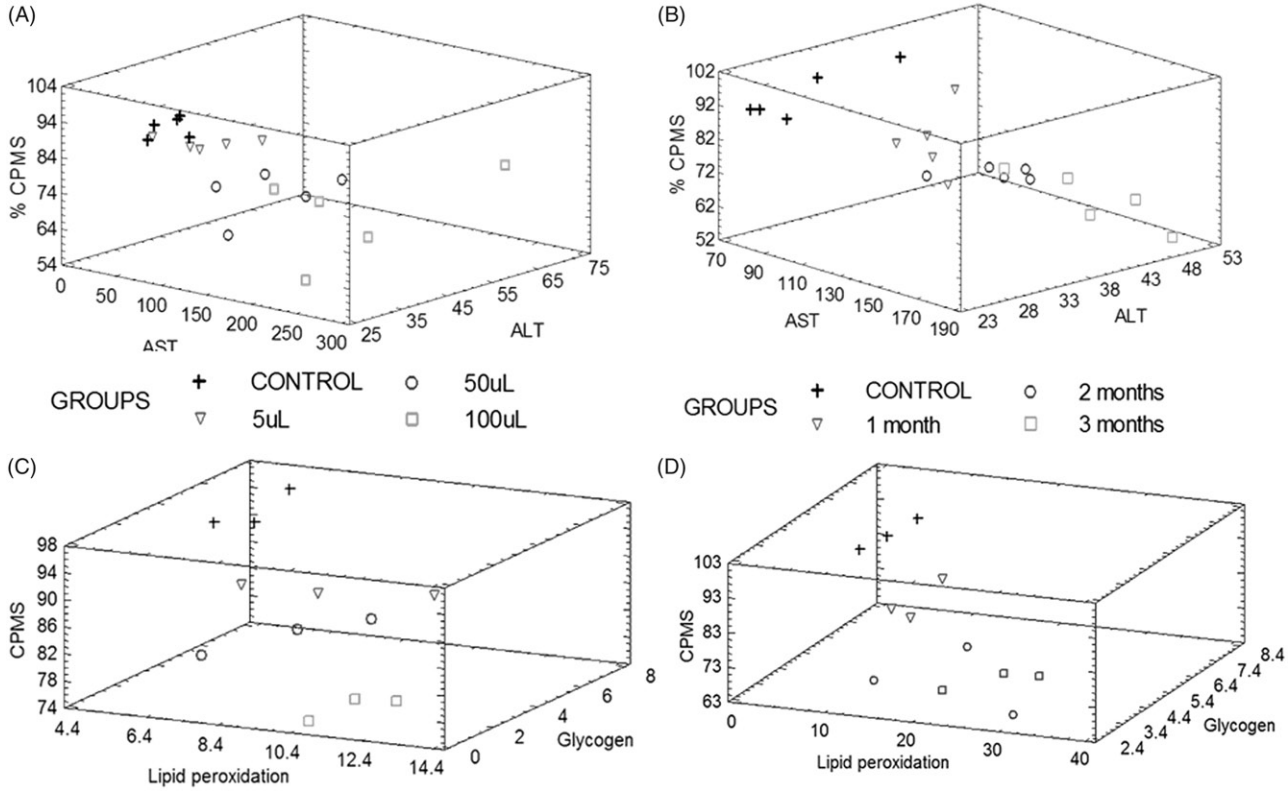

Figure 6. The reduction of CPMS correlated with AST, ALT, MDA, and glycogen levels in liver damage with benzene acute and chronic exposure. Scatter diagram using a discriminant analysis shows individual variability of each data in CPMS values and damage indicators. (A) CPMS and AST/ALT levels in the acute model. Control group shows low aminotransferases levels and high CPMS, while groups with benzene administration show a high aminotransferases levels and low CPMS. Damage for benzene was directly proportional to dose administrated and CPMS decrease. (B) A CPMS and AST/ALT chronic model. Control group shows low aminotransferases levels and high CPMS, while groups with benzene show a high aminotransferases levels and low CPMS. Damage for benzene was directly proportional to exposure time and CPMS decrease. (C) CPMS and liver damage indicators in the acute model. Acute benzene administration groups show decrease in their percentage of CPMS, decrease in glycogen deposits and a high production of MDA in liver. Liver damage for benzene was directly proportional to dose administrated and CPMS decrease. (D) CPMS and liver damage indicators in the chronic model. Control group shows low aminotransferases levels and high CPMS, while groups that were administrated with benzene show high aminotransferases levels and low CPMS. Liver damage for benzene was directly proportional to exposure time and CPMS decrease.

To show CPMS potential as aromatic hydrocarbons indicator we analyzed the relation between CPMS test results with enzymatic, OxS, and glycogen assays in the acute and chronic *in vivo* models, showing a clear correlation between high levels of aminotransferases and MDA as well as glycogen stores decreasing with respect to the administered amount and exposure time to benzene (Figure 6). Based on our results and in the literature (Snyder 2004, 2007) we

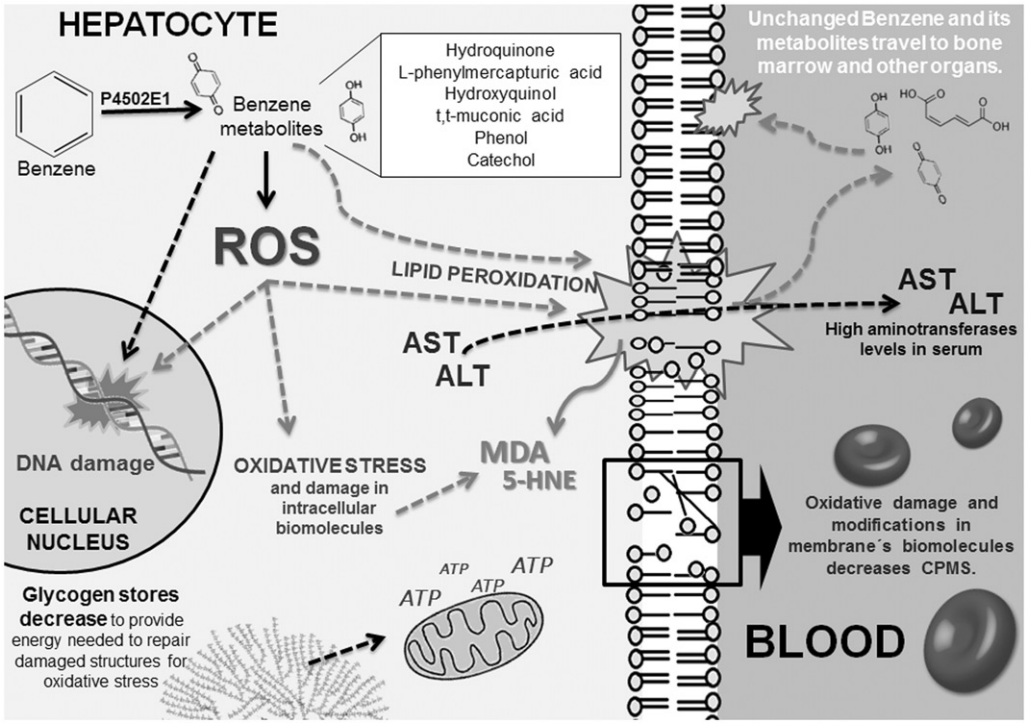

Figure 7. The mechanism of benzene toxicity. In liver and other organs, benzene passes through the cellular membrane because it is a lipophilic substance and it is metabolized by P4502E1 generating different metabolites. ROS are produced by benzene metabolism and their metabolites cause OxS. High concentrations of FR can damage different biomolecules as nucleic acids, proteins, and lipids principally, liberating oxidative products as MDA and 5-HNE. The glycogen stores are diminished for the high cellular energy requirement to induce cell repair. Lipid peroxidation in membrane decreases CPMS and promotes cell death. The damage in membrane allows that aminotransferases, FR, unchanged benzene, benzene metabolites, and other cellular components can go out to blood and travel to other cells.

proposed a mechanism for benzene intoxication. In liver and other organs, benzene passes through the liver cellular membrane and other organs because it is a lipophilic substance. Once it is inside the cells it is metabolized by P4502E1 generating different metabolites like ROS that are produced by benzene metabolism and its metabolites cause OxS. High concentrations of FR can damage different biomolecules as nucleic acids, proteins, and lipids principally, liberating oxidative products as MDA and 5-HNE. The glycogen stores are diminished to induce cell repair by the high cellular energy requirement. Lipid peroxidation in membrane decreases the CPMS and promotes death cell. The damage in membrane allows that aminotransferases, FR, unchanged benzene, benzene metabolites, and other cellular components can go out to blood and travel to other cells (Figure 7).

Discussion and conclusion

Benzene is an aromatic hydrocarbon that enters to body and is metabolized primarily in the bone marrow and liver producing a high amount of metabolites that are sources of FR and OxS (Snyder 2000, 2007; Uzma et al. 2010). All toxic mechanisms of benzene as DNA damage, mitotic inhibition, lipids and proteins damage, and inhibition of enzymes are related to the generation of FR and its metabolites as we proposed in our model (Figure 7) in part supported by literature (Ross 2000; Ibrahim et al. 2014).

AST and ALT are normally cytosolic enzymes involved in the metabolism and upon cellular damage high levels of them are presented in serum (Figure 7). Higher concentrations of AST are found in liver, cardiac muscle, skeletal muscle, kidney, and pancreas. Normally aminotransferase levels are low in the blood. High levels of aminotransferases in blood may be related to damage at the cellular level by necrosis (Gella et al. 1985; Gutierrez et al. 2010). Our results showed that a high aminotransferases concentration in serum related to glycogen, lipid peroxidation, and histological indicators. Dere and Ari reported high levels of aminotransferases in liver benzene toxicity highlighting the importance of AST and ALT as indicator of liver damage (Dere and Ari 2009). Liver glycogen stores decrease with cell damage because cells require energy to be reconstructed and decreases the formation of glycogen (Figure 7) generating structural problems and optimal glucose transporters function (Fong et al. 1953). Our data indicate that glycogen is a good indicator to evaluate hepatic damage for intoxications with benzene. Liver is the organ usually more prone to damage by ischemia and OxS and it is related to aminotransferases level increase in benzene intoxications (Webb and Twedt 2008; Dere and Ari 2009; Sharma and Rana 2010). Therefore, we think that OxS is the bridge between aminotransferases increase and glycogen decrease (Figure 7).

In this study, the level of liver and bone marrow damage is higher, although we found a significant damage in other organs as kidney, spleen, and lungs. Liver and bone marrow metabolized the benzene forming metabolites that are filtered by the kidney therefore harming this organ (Irons et al. 1980). Benzene metabolites have been identified as *trans*, *trans*-muconic acid (*t*, *t*-MA) and are used as biomarkers of benzene in urine (Scherer et al. 1998; Qu et al. 2000;

Wiwanitkit et al. 2001; Wiwanitkit et al. 2007; Ibrahim et al. 2014). As well we found that the spleen showed damage displaying high concentration of MDA and indirectly showed damage in bone marrow because proliferation of megakaryocytes was observed. Normally megakaryocytes grow in bone marrow, but in damage conditions, the spleen is an alternate side of white cells proliferation. DNA damage level has been shown in organs like spleen, bone marrow, liver, and B and T white cells with benzene intoxication in leukemia (Lee et al. 2005). In the case of bone marrow intoxication with benzene, it has been shown that OxS generate genetic aberrations and MDA increases while SOD, catalase, and GSH decrease (Whysner 2000; Healy et al. 2001; Zhang et al. 2002; Holeckova et al. 2004; Whysner et al. 2004; He et al. 2008; Wetmore et al. 2008). The lung showed an inflammation and fibrosis production suggesting that benzene travels for all body through blood and it is metabolized through liver and bone marrow. Benzene metabolites in blood can react with different biomolecules damaging organs with high blood flow as lungs. Several studies indicate that lungs have the capacity to metabolize benzene when it is inhaled (Powley and Carlson 1999; Raj et al. 2001; Powley and Carlson 2002; Park et al. 2009). Our analysis in lipid peroxidation showed a high level of MDA in all organs studied and correlated with histological damage in tissue like liver, kidney, and spleen. The results reported by Verma and Rana in 2004 showed high MDA levels in liver, kidney, and lungs, suggesting that these organs are target of benzene metabolites and OxS. Importantly, we recorder a high MDA level in spleen as well as in brain indicating a generalized toxicological effect.

The CPMS technique was carried out to evaluate oxidative damage in the plasmatic membrane of erythrocytes over OxS conditions where a decrease in the percentage of stability suggests an oxidative damage by FR and benzene metabolites (Figure 7). Compared with healthy cell, a damaged cell shows a decrease in its ability to resist to hemolysis caused by decreasing the extracellular medium isotonicity (Navarro et al. 1993; Brugnone et al. 1999). The isolated erythrocytes plasmatic membranes were subjected to high concentrations of FR generated with benzene metabolism showing a decrease in the percentage of membrane stability dependent of acute intoxication and time-dependent exposure. The results show a relationship between the enzymatic, metabolic, OxS, and histological indicators with the grade of plasmatic membrane damage in erythrocytes, reflecting the level of membrane stability with respect to healthy cells. Interestingly, our results obtained with benzene, toluene, and xylene of CPMS *in vitro* test show dependent damage on dose. Additionally benzene administration in the animal model confirms the utility of this technique to evaluate acute and chronic aromatic hydrocarbons intoxications. Remarkably, CPMS test can be realized with small amount of blood (500 μL minimum), the assay is cheap and fast therefore aromatic hydrocarbons intoxication could easily be evaluated by this methodology.

TBARS assay is the standard technique to determine the level of oxidative damage from biological samples (Ghani et al. 2017). However, in our study CPMS assays show better results in oxidative damage detection compared with the TBARS technique in the acute model, suggesting that the CPMS technique could be more accurate in acute intoxications, since the oxidizing agents induce a considerable plasma membrane damage before there is a high level of necrotic cell death that allows to identify oxidation products in the serum by the TBARS technique. Additionally, AST, ALT, and liver glycogen determinations were made as complementary studies in order to corroborate liver damage caused by benzene. AST and ALT are mainly indicators of liver damage, however they are also present in damage of skeletal muscle, kidney, and pancreas (Johnston 1999; Al-Busafi and Hilzenrat 2013). Interestingly, ALT level was altered with benzene exposure since 100 μL and two months in acute and chronic models, respectively. On the other hand, the level of AST in acute and chronic models increases statistically significant since 50 μL and one month of benzene exposure, respectively, similar to the CPMS level. Therefore, the CPMS technique could be used as a complementary or a substitute test for AST and ALT analyses for liver and kidney damage basing on cheaper and faster assay performance than ALT and AST as well as on similar reliability, sensitivity, and reproducibility. Additionally, CPMS could be used as diagnostic test in exposed persons to aromatic hydrocarbons, for example, organic laboratory, gas station, plastics, and paints industries staff.

The results presented here show that OxS produced by benzene and its metabolites are involved in cell death, inflammation, decreased metabolic function, and the grade of plasma membranes stability, awarding that environmental pollutants as benzene have a general toxic mechanism. The CPMS technique proved to be an excellent indicator of oxidative damage produced by the metabolism of benzene and could be used to design a diagnostic indicator of aromatic hydrocarbons intoxication.

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Disclosure statement

The authors declare no conflict of interest.

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