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Sub-acute Exposure to Benzene Accelerates the Aging Process of Red Blood Cells; an *In vivo* Study

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ABSTRACT

Background: The well-known toxic effects of benzene toxicity are bone marrow depression, reduction in blood cell counts, and induction of leukemia and aplastic anemia. This study was designed to evaluate biomarkers of aging in red blood cells (RBCs).
Methods: Mice were exposed to benzene (50, 100, and 200 mg/kg/day) orally for 28 days. A group of benzene-exposed mice were injected intraperitoneally with N-acetylcysteine (NAC, 150 mg/kg/day). Hematological factors, erythrocyte morphology, and sialic acid content of RBCs along with oxidative stress biomarkers were investigated.
Results: Benzene dose-dependently reduced RBCs count, hemoglobin level, RBCs membrane sialic acid levels, the total antioxidant capacity of plasma, and G6PD activity of RBCs. The activity of antioxidant enzymes and lactate dehydrogenase, oxidative damage end-products and bilirubin levels, reticulocyte count, and RDW and MCV ranges increased in a dose-dependent manner. Poikilocytosis (spherocyte, burr cell, schistocyte and blister cell) and anisocytosis were observed in high doses of benzene.
Conclusion: Our results support the acceleration of RBCs aging and hemolytic anemia in mice exposed to benzene. Co-administration of NAC as an antioxidant effectively alleviated hematotoxicity of benzene.

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Introduction

Industrial air pollutants such as gas fumes, organic vapours, and air particles cause damages in the various tissues.[1] Benzene is an important pollutant in the ambient and indoor air which is widely used as a precursor in the synthesis of many products including drugs, paints, plastics, and pesticides.[2] It has been shown that benzene increases human mortality rate,[3] elevates the risk of respiratory diseases,[4] weakens the immune system, and induces the hematologic effects.[5] There is increasing experimental and clinical evidence that long-term exposure to benzene is associated with hematotoxicity and hematological malignancies.[6] Unlike short-term and acute exposure to benzene, which usually causes neurological complications,[7] long-term exposure to benzene often causes hematologic effects and anemia.[8] Benzene is metabolized to its metabolites (phenol, catechol, hydroquinone) by cytochromes P450.[9] Phenolic metabolites of benzene activate by myeloperoxidase in the bone marrow and produce reactive quinone derivatives.[10] In fact, benzene and its metabolites bind with macromolecules of hematopoietic cells and inhibit the synthesis of red blood cells (RBCs).[11] Previous animal and human studies also showed that the reactive metabolites of benzene covalently binds to the cellular macromolecules in favour of oxidative damages.[12]

No one in the medical field has considered the possibile effect of benzene on RBC aging, which accelerates the

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removal of RBCs. The lifespan of RBCs is 120 days in human[13] and is affected by chemicals and drugs, and liver and kidney diseases.[14] Reduction in metabolic activity of RBCs and morphological alterations including a decrease in cell volume and changes in cell shape happens during the aging process.[15] Changes in membrane fluidity and inactivation of enzymes and membrane-bound receptors during RBCs aging are the results of the oxidation of lipids, and proteins and loss of glutathione (GSH) through inactivation of glucose-6-phosphate dehydrogenase (G6PD).[16] G6PD is involved in the generation of reduced nicotinamide adenine dinucleotide phosphate and reduction of oxidized GSH for the maintenance of cellular redox balance.[17] The decrease in G6PD activity is associated with increased cells oxidative stress and consequently cellular and organism senescence.[18] The membrane sialic acid (SA) is another biomarker of RBCs ageing which the SA content in old RBCs is 10-15% less than in the young one.[19]

The present study aimed to find out the effects of sub-acute exposure to benzene on RBCs aging in mice by emphasis on the evaluation of morphological changes, SA content of RBCs, and oxidative damage biomarkers.

Materials and Methods

Animals

A total of 42 healthy male mice, 8 weeks of age and weighing 22±4 g, were used in this study. The mice were housed in standard cages and given standard mouse pellet and water ad libitum. Experimental protocols are in accordance to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the ethics committee of the neuroscience research centre of Kerman, Iran (code of ethics: IR.KMU.REC.1394.329).

Animal Treatment and Sample Preparation

The mice were randomly divided into seven groups (N=6 per group). The control group received 0.5 ml corn oil orally every day. The treated groups received benzene at the doses of 50, 100, and 200 mg/kg/day of benzene in corn oil by gavage alone or in combination with intraperitoneal N-acetylcysteine (NAC, 150 mg/kg/day) for 28 days. Twenty hours after the end of treatment, blood was taken from the heart and collected in EDTA-coated tubes and clot tube. Blood sample centrifuged at 1000×g for 10 min, 4 °C. The isolated RBCs were washed 4-5 times with phosphate buffer saline (pH 7.4) and lysed with cold distilled water to obtain hemolysate.

Hematology Parameters

Complete blood count (CBC) was determined with the Sysmex cell counter (kx-21N, Japan).

A drop of blood was thinly spread onto a glass slide for preparation of peripheral blood smears (PBS). The slide was then stained with a Wright's stain and cell morphology was analyzed under a light microscope.

Total and direct bilirubin levels were measured by commercial kits (Pishtazteb Co., Tehran, Iran). Indirect bilirubin was obtained by subtracting the direct value from the total value.

For measurement of reticulocyte count, 50 µl of the freshly prepared blood sample was mixed with 50 µl of methylene blue stain (1% in phosphate buffer with pH 6.5) and incubated for 15 min at 37 °C. A wedge-spread film was prepared in a glass slide, and reticulocytes were counted by light microscope among 1000 RBCs. The results were expressed as absolute reticulocyte count (reticulocyte %×RBC count).

Serum Lactate Dehydrogenase (LDH) Activity

The activity of LDH in serum was measured by a commercial kit (Pishtazteb diagnostics Co., Tehran, Iran).

SA Content of RBCs Memberane

RBCs membrane (ghosts) was isolated according to the method explained by Dodge et al.[20] SA of RBCs membrane was measured by the method proposed by Spyridakiet.[21] Briefly, 0.10 ml of 0.04 M periodic acid was added to a glass tube containing 500 µl diluted (20 times) sample and the tube incubated at 0 °C for 30 min. Then, 1.25 ml of resorcinol working solution (5 ml of 6.0% resorcinol, 0.125 ml of 0.1 M copper sulfate, 19.87 ml of distilled water, and 25ml with 10 M HCl) was added. The mixture was heated at 100 °C for 5 min, and after cooling to 0 °C, 3.25 ml of n-butanol was added to the mixture. The n-butanol phase was read at 625 nm, and the SA content was calculated according to the standard curve of N-acetylneuraminic acid.

RBCs Catalase Activity

The catalase activity was measured based on the enzymatic decomposition of hydrogen peroxide (H_2O_2).[22] Briefly, 300 µL of the hemolysate was added to 2.95 ml of 19 mM H_2O_2 in phosphate buffer (50 mM, pH 7.2) and the absorbance was read kinetically at 240 nm for 1 min. The amount of decomposed H_2O_2 was calculated according to its molar extinction coefficient (43.6 M^{-1} cm^{-1}). One µMol decomposed H_2O_2 per min regarded as 1 unit catalase activity.

RBCs superoxide dismutase (SOD) activity

The activity of SOD was measured based on the ability of the sample to inhibit pyrogallol autoxidation.[23] Briefly, 12 µl of distilled water (control) or 12 µl of each sample was added to 32.14 µl pyrogallol (2.6 mM in 10 mM HCl) and 217.85 µl Tris-EDTA buffer (50 mM-1mM, pH 8.2) and was read at 420 nm. The amount of enzyme activity is calculated by this formula: % inhibition of pyrogallol autoxidation=[1-(ΔAbsorbance of sample/ΔAbsorbance of control)]×100.

RBCs G6PD Activity

colorimetric assay kit (BioVision Inc, Milpitas, USA) was used to measure the activity of G6PD.

Total Antioxidant Capacity (TAC)

TAC was determined using the Ferric reducing ability of plasma (FRAP) method which is based on the reduction of $Fe^{3+}$to Fe^{2+}.[24] Briefly, 50 µl hemolysate was added to 1.5 ml

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freshly prepared and pre-warmed (37 °C) FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM 2, 3, 5-triphenyl tetrazolium chloride (TPTZ) in 40 mM HCl, and 20 mM FeCl3.6H2O in a ratio of 10:1:1) and incubated at 37 °C for 10 min. The absorbance was recorded against a reagent blank (1.5 ml FRAP reagent+50 µl distilled water) at 593 nm. Ferrous sulfate standard curve was used to calculate the amount of Fe^{2+}.

Lipid Peroxidation

Malondialdehyde (MDA) as a lipid peroxidation end product, was assessed according to thiobarbituric acid reactive substances.[25] To carry out this experiment, 100 µl hemolysate was was added to 1 ml treated with TCA 20% and centrifuged at 1000×g for 5 min. One ml of supernatant was mixed with 1 ml thiobarbituric acid (0.67%, pH 7.4) and incubated at 100 °C for 20 min and Then, 1 ml n-butanol was added. The absorbance of the extracted colour was measured at 535 nm and the amount of MDA were calculated according to MDA standard curve.

Protein Carbonylation

The protein carbonyl groups were measured by the method of Levine et al.[26] Briefly, 100 µl hemolysate was added to 1 ml trichloroacetic acid (TCA, 20%) and the pellet was resuspended in 0.5 ml 2,4-dinitrophenylhydrazine (DNPH, 10 mM) and allowed to stand at room temperature for 60 min with vortexing every 15 min. The proteins were precipitated again with TCA 20% and the pellet was washed three times with 1 ml ethanol: ethyl acetate (1:1 v/v) solution. Finally, the pellets were dissolved in 0.8 ml of 6 M guanidine hydrochloride at 37 °C and centrifuged at 5000×g for 5 min and the supernatant was read at 370 nm. The carbonyl group content was calculated using the molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

Statistical Analysis

GraphPad Prism v6.01 software (GraphPad Software, Inc., San Diego, CA, USA) was used for the statistical analyses in this work. Statistical analyses were done using Student's *t*-test and by one-way ANOVA. P<0.05 was considered as the significance level for all the tests.

Results

Hematological Findings

CBC results showed a dose-dependent reduction in RBC and WBC count and hemoglobin level as well as the elevation in MCV, RDW, and reticulocyte count in the mice treated with different doses of benzene. The hematological parameters did not change in benzene-treated mice received NAC, especially at lower doses of benzene. Benzene at the doses of 100 and 200 mg/kg/day significantly increased the indirect bilirubin, but it did not happen in the mice exposed to benzene treated with NAC (table 1).

Morphological abnormalities of RBCs were increased in a dose-dependent manner (table 2). A few burr cells were observed in the group treated with 50 mg/kg/day benzene, and other forms of abnormal RBCs including spherocyte, schistocyte, anisocytosis, and poikilocytosis were also observed after treatment with benzene at the dose of 100 mg/kg/day. In addition to the presence of dacrocytes and blister cells, the morphological changes of RBCs were more severe after exposure to 200 mg/kg/day. Treatment with NAC in the mice received benzene at the doses of 50 and 100 mg/kg/day eliminated morphological abnormalities thoroughly, but a mild abnormal RBCs including burr cells and anisocytosis remained in the group received 200 mg/kg/day benzene after treatment with NAC.

Serum LDH Acitvity

Benzene at the doses of 50, 100, and 200 mg/kg/day significantly increased the activity of serum LDH in the benzene-treated mice. NAC reduced serum LDH in the benzene-treated mice at the dose of 50 mg/kg/kg but not at the doses of 100 and 200 mg/kg/day (figure 1a).

Table 1: Complete blood count, reticulocyte count, and serum indirect bilirubin level in the control group, benzene-treated group, and benzene-treated group received N-acetylcysteine after 28 days of treatment

Variables	Control	Benzene (mg/kg/day)			Benzene (mg/kg/day)+NAC (mg/kg/day)		
		50	100	200	50+150	100+150	200+150
RBC	7.18±0.92	5.48±0.52	4.27±0.46 [b]	3.45±0.26 [c]	6.45±0.59	5.15±0.53	4.98±0.20 [a]
WBC	1.93±0.53	1.16±0.22	1.08±0.38 [c]	1.56±0.72 [c]	2.37±0.42 [b]	2.30±0.26 [c]	2.26±0.27 [c]
HB	14.89±1.29	12.03±0.39 [c]	10.78±0.36 [c]	9.38±0.37 [c]	15.73±0.19	13.98±0.50 [b]	13.10±0.79 [c]
HCT	46.63±1.81	44.56±1.27	42.36±1.03	41.76±1.52 [a]	44.96±0.97	43.91±1.40	42.93±1.19 [a]
MCV	51.33±1.59	53.33±0.88 [c]	55.56±1.35 [c]	58.90±0.294 [c]	50.79±0.79	51.383±1.02	53.95±1.59 [a]
MCH	15.16±0.80	13.41±0.61	13.63±0.71	13.39±0.63	14.65±0.62	13.45±0.54	13.93±0.36
MCHC	31.80±1.01	31.08±0.67	29.90±0.61	27.95±0.32	30.89±0.76	30.92±0.69	29.78±0.77
PLT	1386.00±92.84	1274.80±60.10	1273.16±70.40	1241.30±79.62	1270.16±77.30	1285.00±67.05	1312.80±74.30
RDW	15.26±0.6 0	16.21±0.52	16.91±0.30 [a]	17.93±0.20 [c]	15.46±0.35	16.10±0.28	15.80±0.48
Reticulocyte	2.50±0.83	4.10±0.69	6.70±0.52 [b]	10.90±0.20 [c]	2.60±0.58	3.5±0.47	5.10±0.20 [b]
Indirect bilirubin	0.27±0.19	0.80±0.36 [b]	1.32±0.31 [c]	2.05±0.68 [c]	0.31±0.15	0.50±0.44 [a]	0.98±0.56 [b]

Red blood cell (RBC; $\times10^6/\mu L$), white blood cell (WBC; $\times10^3/\mu L$), hemoglobin (HB; g/dl), hematocrit (HCT; %), mean corpuscular volume (MCV; fl), mean corpuscular hemoglobin (MCH; pg/cell), mean corpuscular hemoglobin concentration (MCHC; g/dl), platelet (PLT; $\times10^3/\mu L$), RBC distribution width (RDW; %), reticulocytes (%), N-acetyl cysteine (NAC). Data are shown as mean±SD and the number of mice in each group was 6. Results with P<0.05 was considered significant. [a] P<0.05; [b] P<0. 01; [c] P<0.001

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Table 2: Morphology of red blood cells in the control group, benzene-treated group, and benzene-treated group received N-acetylcysteine after 28 days of treatment

Variables	Control	Benzene (mg/kg/day)			Benzene (mg/kg/day)+NAC (mg/kg/day)		
		50	100	200	50+150	100+150	200+150
Dacrocyte	-	-	-	+	-	-	-
Blister cell	-	-	-	+	-	-	-
Schistocyte	-	-	+	+++	-	-	-
Burr cell	-	+	+	+++	-	-	+
Spherocyte	-	-	+	+++	-	-	-
Poikilocytosis	-	-	++	++	-	-	-
Anisocytosis	-	-	++	+++	-	-	+

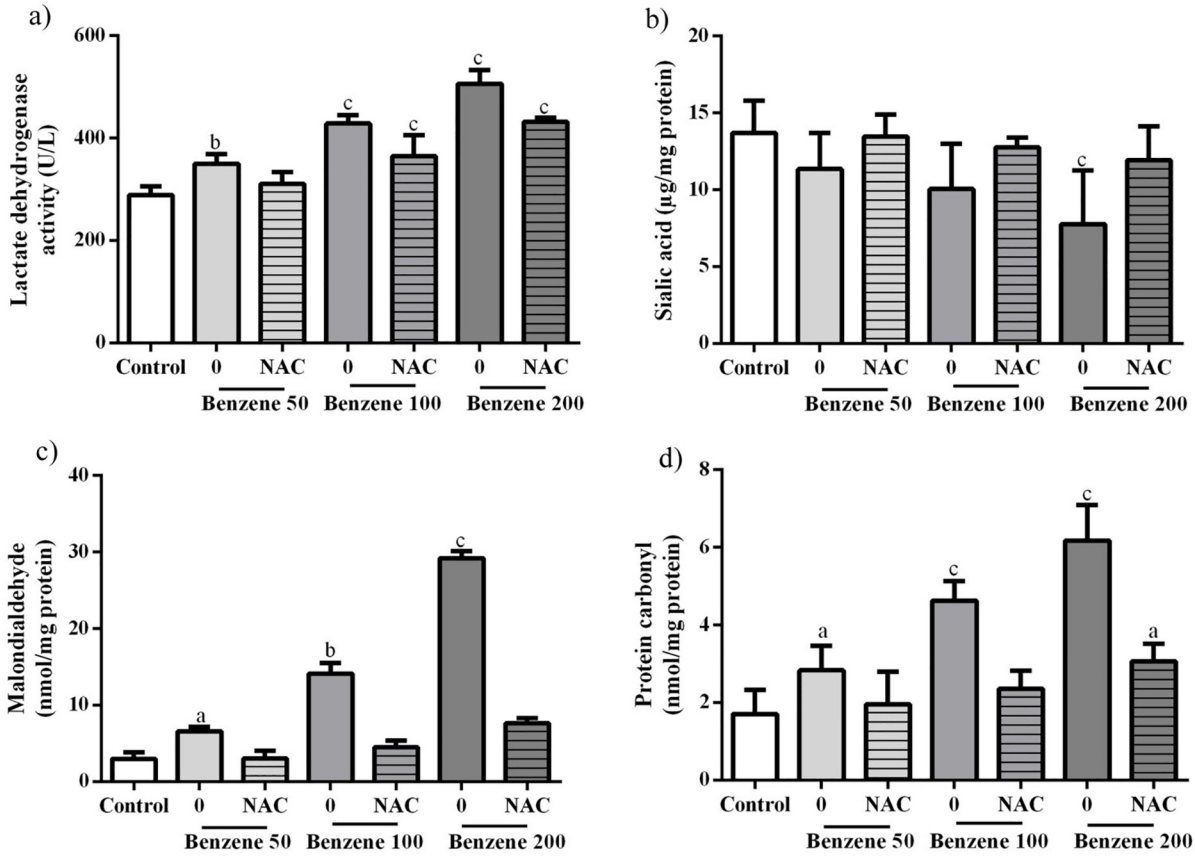


Figure 1: a) Lactate dehydrogenase activity, b) membrane sialic acid content, c) lipid peroxidation as malondialdehyde level, and d) protein carbonyl content of red blood cells in the control group, benzene-treated groups (50, 100, and 200 mg/kg/day), and benzene-treated groups received N-acetylcysteine (NAC, 150 mg/kg/day) after 28 days of treatment. Data are shown as mean±SD and the number of mice in each group was 6. Results with P<0.05 was considered significant. [a] P<0.05; [b] P<0.01; [c] P<0.001.

SA Content

As shown in figure 1b, the SA level decreased in the erythrocyte membrane of mice treated with 200 mg/kg/day benzene. Reduction of SA prevented by treatment of benzene-exposed mice with NAC. Sub-acute exposure to the lower dose of benzene did not significantly reduce the content of SA in RBCs.

Oxidative Stress Biomarkers

The activity of antioxidant enzymes is presented in table 3. Results showed a reduction in the activities of catalase and SOD in groups treated with benzene compared to the control group. The activity of these enzymes did not change in the benzene-poisoned mice treated with NAC. Benzene significantly reduced the G6PD enzyme activity in RBCs at the dose of 200 mg/kg/day, and NAC did not significantly increase the activity of this enzyme in mice received 50, 100 and 200 mg/kg/day benzene (table 3). Benzene at the dose of 200 mg/kg/day reduced the plasma antioxidant capacity, and NAC elevated plasma antioxidant capacity in mice treated with benzene (50 and 100 mg/kg/day).

Sub-acute exposure to benzene in all three doses increased the concentration of MDA, the end product of lipid peroxidation, which was significantly reduced by NAC (figure 1c). Protein carbonyl levels increased in benzene-treated mice but not in those groups received NAC (figure 1d).

Discussion

In this study, sub-acute exposure to benzene reduced RBCs count which it could be as a result of decrease

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Table 3: Antioxidant capacity of red blood cells in the control group, benzene-treated group, and benzene-treated group received N-acetylcysteine after 28 days of treatment

Variables		Control	Benzene (mg/kg/day)			Benzene (mg/kg/day)+NAC (mg/kg/day)		
			50	100	200	50+150	100+150	200+150
Enzymatic antioxidants	CAT	0.25±0.06	0.13±0.06	0.14±0.09 [a]	0.16±0.20 [c]	0.26±0.07	0.29±0.08	0.38±0.04 [b]
	SOD	0.97±0.17	0.61±0.17 [c]	0.67±0.17 [c]	0.41±0.23 [c]	1.02±0.18 [c]	1.60±0.18 [c]	1.15±0.18 [c]
	G6PD	1.54±0.49	1.36±0.503	1.23±0.26	0.62±0.21	1.62±0.72	1.51±0.38	0.87±0.65
FRAP		1.29±0.30	1.03±0.23	0.96±0.08	0.78±0.04 [b]	1.40±0.18 [a]	1.30±0.04	1.19±0.35 [a]

Catalase (CAT; mU/mg protein), ferric reducing antioxidant power (FRAP; nMolFe2+/mg protein), N-acetylcysteine (NAC), glucose-6-phosphate dehydrogenase (G6PD; nMol/min/mg protein), superoxide dismutase (SOD; U/mg protein). Data are shown as mean±SD and the number of mice in each group was 6. Results with P<0.05 was considered significant. [a] P<0.05; [b] P<0. 01; [c] P<0.001

in the production of RBCs and/or the acceleration of RBCs aging process. NAC as a free radical scavenger reduced the toxic effects of benzene on RBCs at the doses, but not at the dose of 200 mg/kg/day because of extensive oxidative damage of RBCs. the decrease in the hemoglobin and hematocrit and the increase in peripheral blood reticulocytes, MCV, and RDW in the benzene-treated groups could be secondary to a reduction in the RBCs count. Ward and et al. reported similar results in sub-chronic exposure to benzene.[27] Elevation in MCV occurred after sub-chronic exposure to benzene in Chinese workers.[28] Presence of schistocytes and blister cells in the peripheral blood smear is indicative of hemolysis.[29] Anisocytosis also reported in a model of the inhalational toxicity of benzene on mice.[30] On the other hand, since NAC decreases oxidative damages due to the improvement of glutathione deposits, anisocytosis was reduced and morphological changes were not observed in RBCs of benzene-exposed mice treated with NAC.[31] The findings of CBC and morphological data (especially elevation in the number of schistocytes, spherocytes, and blister cells) support the possibility of an increase in the destruction of RBCs. Benzene typically causes aplastic anemia[32] and pernicious anemia,[33] and the results of this study proposed occurrence of hemolytic anemia. This study showed that benzene increased the enzyme activity of LDH and indirect bilirubin levels in serum which also reported in other studies.[34] Increased levels of indirect bilirubin and LDH in serum could be related to hemolysis in samples treated by benzene.[35]

In this study, the increase in the lipid peroxidation and protein carbonylation of RBCs and the decrease in the total antioxidant capacity of plasma and the activity of SOD, catalase, and G6PD in RBCs are indicative of oxidative damage caused by benzene that effectively eliminated by NAC. Oxidative stress is a physiological process which is induced by overproduction of reactive oxygen species (ROS). ROS are produced endogenously during normal cellular metabolism and play a crucial role in regulating normal cell growth by modulating redox-sensitive signalling pathways.[36] Excessive production of ROS along with disorder in cellular antioxidant defense systems in pathological situations destroy cellular homeostasis and membrane integrity through oxidative damage of macromolecules.[37] Production of ROS during autoxidation of hemoglobin as the oxygen carrier protein contributed to RBCs aging.[38] Studies have shown that elimination of G6PD activity as an antioxidant contributed to oxidative damages of RBCs and acceleration of the RBCs aging process.[17, 39] Reduction in the membrane SA content of RBCs in the benzene-treated group accelerates RBCs aging and lysis of cells which can be attributed to the elevation of oxidative damage of RBCs.[40]

Conclusion

Taking together, morphological alterations in RBCs (schistocytes, spherocyte), changes in the size of RBCs (anisocytosis and poikilocytosis), reduction of RBCs count, and the increase in reticulocytes count could raise suspicion of hemolytic anemia which confirmed by the elevated level of bilirubin and LDH in serum. The findings of this study suggest that one of the mechanisms involved in the hemathological effects of benzene is the induction of oxidative stress and acceleration of the aging process in RBCs. Another issue that comes to mind in this regard is the direct interaction of benzene and its metabolites with SA moieties on surface glycoproteins of RBCs membrane that can be studied further.

Conflict of Interest: None declared.

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