International Proceedings of Chemical, Biological and Environmental Engineering, V0l. 90 (2015)
DOI: 10.7763/IPCBEE. 2015. V90. 18

Effect of Inhaled Benzene on Mice's Peripheral Blood: Erythrocyte Morphology & Count Analysis, Haematocrit & Haemoglobin Level and Erythrocyte Indices

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Abstract. Benzene is a well-known carcinogen that results in haematotoxicity. This study was conducted to investigate the effect of inhaled benzene on mice's peripheral erythrocytes based on its morphology, count, haematocrit and haemoglobin level and erythrocyte indices analysis. A total of 32 mice were divided into two groups, (1) expose to benzene and (2) non-exposed group. After 4 weeks of exposure through inhalation, blood samples were drawn from anesthetized mice at orbital sinus. Collected blood samples were subjected for morphological analysis, erythrocyte count, haematocrit and haemoglobin level and erythrocyte indices analysis. Upon exposure, the appearance of dacrocytes, accompanied by microcytosis and acanthocytes was noted. Significant increases were recorded for erythrocyte count, haemoglobin level and mean cell volume (MCV) calculation. However, no significant changes were noted for haematocrit, mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) calculation. Increase in erythrocyte appeared to serve as a filler, preventing empty space after the loss of bone marrow cells. In conclusion, 4 weeks exposure of benzene through inhalation causing alteration in erythrocytes morphology and count, haemoglobin and haematocrit level in mice.

Keywords: Benzene, Carcinogen, Hematotoxicity, Erythrocyte.

1. Introduction

Benzene, an aromatic hydrocarbon, has been widely used as a multipurpose organic solvent. It is a natural constituent of gasoline and found in mainstream cigarette smoke [1], [2]. Chemical intermediate of benzene is also been used during syntheses of many pesticide and pharmaceutical products [3] resulting in a significant potential for human exposure. The use is now discouraged due to its high toxicity, including carcinogenicity. International Agency for Research on Cancer has classified benzene as one of the group 1 carcinogen [4].

Benzene is colourless and easily evaporates. Its vapour was heavier than air and may sink into low-lying area. Due to this characteristic, many people inhaled benzene without knowing it. Indoor air generally contains higher level of benzene than those in outdoor air. The source of benzene in indoor air comes from glues, paints, furniture wax and detergent. The emission from burning coal, gasoline fumes, automobile exhaust, cigarette smoke and wood-burning fires are the source of benzene from outdoor air. Exposure to benzene has become common recently as more people have moved into newly constructed apartments and done lots of indoor decoration for improvement of their living conditions. This statement was support by

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study by Juan *et al*. (2010) [5]. They reported that vapours from decoration materials such as furniture wax, detergents, glues and paints were the main sources of exposure.

The primary toxicology effects of chronic benzene exposure are on the hematopoietic system. It is a ubiquitous pollutant with known hematologic and carcinogenic effects in human and mice [6]. Tunsaringkarn *et al*. (2013) performed a study on 102 gasoline station workers from 11 gasoline stations, examining the correlation between the urinary levels of benzene metabolites with haemoglobin, haematocrit, white blood cell and platelet count. These researchers found a significant reduced in haemoglobin, haematocrit and eosinophil count, that may associated with bone marrow depression [7]. Severe benzene exposure in low-level is also believed can lead to life-threatening disorder. In 1999, a 45-year-old petrochemical worker was referred to St. Mary's Hospital, The Catholic University of Korea. Based on the case report, the patient was exposed to low concentration of benzene for about 21 years due to the nature of his work, packaging powder resin into bags. A biopsy bone marrow was taken and result showed hypocellularity with fatty infiltration, which is consistent with aplastic anemia [8].

A number of animal studies have demonstrated that benzene exposure can induce in bone marrow damage and changes in circulating blood cells in general [9]-[14]. Most of it reported the occurrence of leukopenia, increased spleen weight and histological changes to the bone marrow which include depressed marrow cellularity and reduced stem cell count. However, very limited study focuses the toxic effect on erythrocyte and its associated parameter. Thus, this paper aims to examine the effect of inhaled benzene on mice's peripheral erythrocyte morphology and count, haematocrit and haemoglobin levels and the erythrocyte indices.

2. Methodology

2.1. Animal Preparation

We obtained approval from our Institution's Research and Ethics Committee. The usage of animals for this project has been approved by the Institution's Animal Ethics Committee. A total of healthy 32 male BALB/c mice, averaging at 8 weeks of age and weight approximately 20-30 g were used in this experiment. The mice were maintained under controlled temperature (21-23 ˚C) and 12h light/12h dark cycle conventional condition with free access to food and water for at least one week prior to experimental procedure. The mice used were divided into two groups which are: 1) expose to benzene group (n=16) and 2) non-exposed group (n=16). The groups which exposed to benzene were subjected to daily benzene inhalation for 6 hours/day, 5 days/week for subsequently 4 weeks - which each of group was given same volume of benzene. The exposure test was carried out in clear transparent plastic boxes to generate a constant airstream from an aqueous solution of benzene. A commercially available 99.8%, anhydrous, benzene solution was used in this study. The weight and physical observation of mice was recorded in daily basis.

2.2. Blood Collection

Upon exposure period, 2 ml of blood was collected via orbital sinus. The blood collection procedure was conducted under anesthesia using diethyl ether. The mice were scuffed with thumb and forefinger of the non-dominant hand and the skin around the eye was pulled. A capillary tube was inserted into the medial canthus of the eye (30 degree angle to the nose). Once the plexus or sinus was punctured, blood that came out through the capillary tube was collected in EDTA tube. Once the require volume of blood was obtained, the capillary tube was gently removed and wiped with sterile cotton. Bleeding was stopped by applying gentle finger pressure [15].

2.3. Hematological Analysis

Collected blood was subjected for hematological analysis which includes erythrocyte morphology and count, hematocrit and hemoglobin level and erythrocyte indices; mean corpuscular volume of single red cell (MCV), mean corpuscular hemoglobin weight (MCH), mean corpuscular hemoglobin concentration (MCHC).

Blood smear was prepared and stained with Wright-Giemsa staining. Stained slides were examined under light microscope for morphological changes in erythrocyte [16]. Total number of erythrocytes was counted manually using hemacytometer at 40 x objective lens. Erythrocytes were counted in the five designated squares [17]. Hematocrit level was measured following method describe by Walker et al. [18]. Well-mixed anticoagulated bloods were drawn into microhematocrit tube by capillary action. The other end of the capillary tube was sealed with a small amount of clay material. The sample was centrifuged for 5 minutes and was read by using the hematocrit reader. Hematocrit level was calculated using a standard formula. Drabkin method was used to measure the hemoglobin level. This method involved the preparation of standard hemoglobin curve between 4 to 20 g/dL hemoglobin concentration. The standard solution was read using spectrophotometer at 540 nm. A hemoglobin standard curve was plotted using a graph paper. The same procedure was repeated for samples and the hemoglobin concentration was extracted from the prepared standard curve [19]. The erythrocyte indices were calculated following method described by Walker et al. [18].

2.4. Statistical Analysis

All statistical analysis was carried out using Statistical Package for the Social Science (SPSS) statistical software version 20. The collected data was expressed in mean ± standard error of the mean (S.E.M). Student t-test was used to compare means among two experimental groups. The data were considered as significant when p value is less than 0.05.

3. Results

3.1. Effect of Benzene on Erythrocyte Morphology

Exposure to benzene altered the shape and size of the erythrocyte. Figure 1 showed the morphology of exposed and non-exposed erythrocyte. In non-exposed group, the shape and size of the erythrocyte remained normal. They appear as biconcave cell with center pallor. The size recorded was 8 micron. However, variation in shape (poikilocytosis) and size (anisocytosis) was observed in exposed group. Dacrocytes and acanthocytes were significantly appeared. Also noticed during the observation are some degrees of microcytic cells.

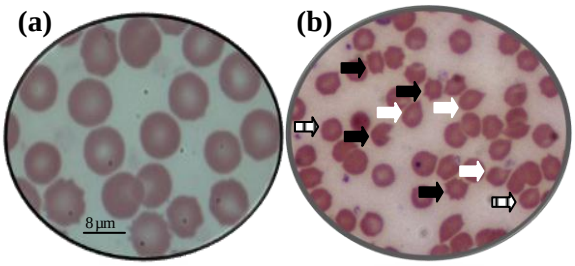

Fig. 1: Morphology observation of (a) non-exposed and (b) exposed group. The erythrocyte shape, size and color of non-exposed group remained normal; biconcave shape with center pallor and 8 micron in size. However, exposed groups revealed the appearance of dacrocyte (tear-drop cell, white arrows) and acanthocytes (fragmented cell, black arrows). Microcytic cells were also noted in exposed group (line arrows).

3.2. Effect of Benzene on Erythrocyte Count, Hemoglobin and Hematocrit Level

Erythrocyte count analysis was conducted manually via hemocytometer. In the present study, significant increase of erythrocyte count (Table 1) was observed in exposed group (23.88×10^{12}/L ± 0.89) as compared to non-exposed group (14.19×10^{12}/L ± 0.22). Correspond to the increase in erythrocyte number, mean hemoglobin and hematocrit was also increased. In exposed group, 23.88g/dL of hemoglobin was detected as compare to non-exposed group, 14.19 g/dL. Hematocrit level was 38.19% in exposed group and 35.37% in non-exposed group. In general, it is 63.3% increase in erythrocyte count, 41% of increment in mean hemoglobin and 7.4% of increment in mean hematocrit upon the exposure towards benzene.

Table 1: Erythrocye count analysis, hemoglobin and hematocrit level of non-exposed and exposed group. Significant increase of erythrocyte cell count and hemoglobin level was seen in exposed group as compared to non-exposed group.

	Non-exposed	Exposed
Erythrocyte Count ($x10^{12}$/L)	14.19 ±0.22	23.88 ±0.89**
Hemoglobin (g/dL)	14.19	23.88*
Hematocrit (%)	35.37	38.19

Different from non-exposed: *p< 0.05, **p<0.01

3.3. Erythrocyte Indices Analysis

Upon getting the erythrocyte count, hemoglobin and hematocrit level, the erythrocyte indices can be calculated. On average, the MCV in exposed group are lower *(M = 1.8 fL, SE = .10)* than in non-exposed group *(M = 4.6 fL, SE = .26).* This different was significant $t(15) = 9.946$, $p < .001$. In contrast, the MCH in exposed group are higher *(M = 5.06 pg, SE = 1.41)* than in non-exposed group *(M = 2.26 pg, SE = .32)* and the MCHC in exposed group are higher *(M = 62.46 g/dL, SE = 4.55)* than in non-exposed group *(M = 50.36 g/dL, SE = 8.17)*. However, this different was not significant [$t(16) = 1.932$, $p = .091$; $t(16) = 1.294$, $p = 0.222$] respectively. Generally, exposure to benzene reduced 61% of MCV value, increase 55% of MCH value and increase 19% of MCHC value when compare to non-exposed group.

4. Discussion

Benzene is a ubiquitous environmental pollutant being used not only for industrial purposes, but also the constituent of daily product such as detergent, paint and furniture wax. Depending on the amount and duration of exposure, benzene can cause serious negative health effects to human being. The ability to conjugate, results in the elevation of benzene metabolites interactions that may further increase its toxicity [20]. The reactive intermediates of benzene are able to bind to cellular macromolecules to induce damage [21]. In addition to this, it has been shown benzene exposure affect many organ such as kidney, liver, brain, as well as bone marrow, causing blood and blood components changes [22], [23].

Previous study conducted by Rothman *et al.* [24] showed that exposure to benzene reduced the total white blood cell, absolute lymphocyte count, platelets, erythrocyte count and hematocrit level causing anemia among workers exposed to > 30 ppm of benzene. However, in the present study indicates on the contrary that there is an increase of erythrocyte count among the experimental mice exposed to benzene. This is supported the study conducted by Uzma *et. al.* [25]. They showed that exposure to benzene and other air pollutant like carbon monoxide (CO) among the petrol filling workers, gradually increase the red blood cells (RBCs) count and hemoglobin level. According to Williams *et al.* [26] exposure to CO is causing tissue hypoxia and eventually lead to a stimulation of RBCs formation. CO was emitted from internal combustion engines of motor vehicles and enters the blood through respiratory system. CO possess the ability to binds over 200 times more firmly to hemoglobin than oxygen, forming carboxy hemoglobin and interfering with blood's oxygen transport capability, results in hypoxic hypoxia. Tissue hypoxia is the potent stimulus for the RBCs production, so it leads to the stimulation of erythropoietin (EPO) – a factor which stimulates the erythropoiesis (RBCs production).

Besides, metabolisms of benzene produce its metabolite, Quinone. Quinone is capable to give rise to reactive oxygen species (ROS). ROS is also formed due to abnormal condition like hypoxia [27]. Hypoxia will stimulate kidney and liver to produce more EPO, the glycosylated protein hormone for erythropoiesis. This scenario ultimately leads to the production of more number of erythrocyte and hemoglobin in the circulating blood. Theoretically, increase in erythrocyte count will lead to increase in hemoglobin level. It was known that the primary function of hemoglobin residing within erythrocyte is to bind oxygen and transport the oxygen to body tissues. Thus, large amount of hemoglobin was consistent with the requirement for large oxygen transport capacity.

Marked increase of erythrocyte count may also result from the effect of benzene towards bone marrow. Farris *et al.* [28] reported that benzene reduced the number of total bone marrow cells, progenitor cells, and differentiating hematopoietic cells in mice exposed to 100 and 200 ppm benzene. Replication of primitive progenitor cells in the bone marrow was increased during the exposure period as a compensation for the

cytotoxicity induced by 100 and 200 ppm benzene. Relatively, the increase in erythrocyte appeared to serve as filler, that preventing empty space after the loss of hematopoietic cell.

There was 7.4% increment of mean hematocrit after the exposure. It is believed that such increase was due to increase number of erythrocyte in circulation, although the increase was not significant. Hematocrit is the volume percentage of erythrocyte in blood. Measurement of hematocrit is primarily depends on the number of erythrocyte and secondarily to other factors like plasma volume and size of erythrocyte. The value of hematocrit is proportional to the number of erythrocyte. In this study, 63.3% increment of erythrocyte leads to slight increment of hematocrit, most probably due to the above mentioned fact. As the body weight of the experimental mice was maintained at normal stage (water and food consumption gradually increase proportional to the body weight) and the experimental mice used maintained healthier (data not shown) throughout the experiment, it is believed that the plasma volume was also at normal stage. Thus, increase in erythrocyte number might be the potential contributor for increase in hematocrit level.

Results of the present study indicate that benzene provoked an alteration of experimental mice erythrocyte morphology from the normal discoid shape to other forms such as dacrocytes and acanthocytes. Dacrocytes is characterized by the cell in shape of tear drop. Usually, these shapes are accompanied by microcytosis (smaller in size). Acanthocytes or spur cells have many tiny spicules that unevenly distributed over cell. It is also noticed as fragmented cells under the microscope. Structural defect and changes in surface shapes of erythrocytes have been reported by Zeni *et al.* [29] from anionic detergent (sodium dodecyl benzene sulphonate) exposed fish. It is believed that acanthocytes may result from cellular adaptation of physiological parameters involved in shape maintenance. Also, it can be speculated that benzene and/or its metabolite caused alterations in the cytoskeleton (membrane proteins and/or lipids) of erythrocytes thus affecting the surface area of the cell.

Calculation of the erythrocyte indices is generally regarded as the initial indicator for the diagnosis of types of anemia. Anemias are defined based on the cell size (MCV) and amount of hemoglobin (MHC). In the present study, it was found that there was a significant reduced in the MCV level, but the MCH and MCHC levels were only slight increase when comparing to non-exposed group. Reduce in the cellular size could be due to the membrane alterations. Benzene may have impact on the cell flexibility and permeability by mechanism not yet defined. A reduced of erythrocyte size (MCV) has been associated with several factors but it is generally considered as a response to stress (most probably due to the alteration in the cytoskeletons). Further investigation on the mechanism could be important for better understanding.

MCH and MCHC reflect the hemoglobin content of the erythrocytes. MCH indicate the amount of hemoglobin in each of the erythrocytes, while MCHC is the amount of hemoglobin relative to the size of the cell per erythrocyte. An increase in MCHC can be a useful clinical indicator of increased spherocytes (spherocytosis), as in hereditary spherocytosis, autoimmune hemolytic anemia or hemoglobin C disease [30]. In contrast to the result in present study, no spherocytosis was noticed upon exposure to benzene, although the hemoglobin content was increased. It is believed that the increase in hemoglobin content is mainly due to the increase of erythrocyte numbers.

5. Conclusion

Under the light of this study, it is concluded that benzene is causing alteration in erythrocytes morphology and count, hemoglobin and hematocrit level. Even though limitations exist to extrapolate experimental results in animal to human, this study might be useful in conducting in vitro investigations with human erythrocytes for better understanding on the possible deleterious effects of the benzene towards erythrocytes structural and numbers alteration, hemoglobin and hematocrit level.

6. Acknowledgements

We would like to thank the Institute of Medical Science Technology for funding this study. We are grateful to Hazwani Mohd Yusuf and Noramirah Mohamad @ Yusoff for scientific and technical assistance. We also would like to thank Mr. Mohd Rohani Jais and the team for their assistance in laboratory works.

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