INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-7, Issue-1, Jan-Mar-2016 Coden IJABFP-CAS-USA Copyrights@2016

Received: 6th Dec-2015 *Revised: 26th Dec -2015* *Accepted: 29th Dec -2015*

Research article

INDUCTION OF APLASTIC ANEMIA IN EXPERIMENTAL MODEL

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ABSTRACT: Aplastic anemia is a life-threatening disease characterized by hypocellular marrow and pancytopenia as a result of reduction in hematopoietic progenitor and stem cells. Secondary aplastic anemia occurs after exposure to environmental factors and in certain disorders. The factors which have been implicated as causes of secondary aplastic anemia are chemicals, drugs, infectious agents, radiation and rheumatic disease.

This study aimed to present a model of induced aplastic anemia in mice by benzene intoxication subcutaneously or orally. Male CD1 mice were used as experimental animal. These animals were classified into four groups as follow:

1- ***Control group***, received only the ordinary mice diet and water and administered with 2ml/Kg saline subcutaneously (sc) daily along the time of experiment.
2- ***Sc day after day treatment group*** with 2ml/Kg of benzene for 15 dose (i.e. for 30 days)
3- ***Sc daily treatment group*** with 2ml/Kg of benzene for 15 dose (i.e. for 15 days)
4- ***Oral daily treatment group*** with 2ml/Kg of benzene for 15 dose (i.e. for 15 days)

The study concluded that, by benzene intoxication, hematological parameters in peripheral blood and bone marrow was affected as follow: 1-Reduction in blood cell counts was occurred, in RBCs, WBCs, platelets, and hemoglobin. Lymphocytes percentages in blood were depressed and neutrophils percentages were elevated in all intoxicated groups. 2- Bone marrow depression was occurred by benzene as a reduction in bone marrow cellularity and slow rate of cells maturation.

Key words: Aplastic anemia, Benzene, Mice, Experimental model

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INTRODUCTION

Aplastic anemia is a life-threatening disease characterized by hypocellular marrow and pancytopenia as a result of reduction in hematopoietic progenitor and stem cells. Usually, aplastic anemia is a result of hematopoietic progenitor and stem cells destruction targeted by autoreactive cytotoxic T cells. Oligoclonal expansion of T-cell receptor Vb subfamilies and interferon gamma can be detected in peripheral blood mononuclear cells of these patients. Although many factors have been implicated in autoreactive T-cell activation, no conclusive causes have been identified. In, 10% of aplastic anemia patients, the disease mechanism has a genetic basis with inherited mutations or polymorphismin genes that repair or protect telomeres. These defects result in short telomeres, which dramatically decrease the proliferative capacity of hematopoietic progenitor and stem cells (Young et al., 2006 and 2010).

Secondary aplastic anemia occurs after exposure to environmental factors and in certain disorders. The factors which have been implicated as causes of secondary aplastic anemia are chemicals, drugs, infectious agents, radiation, rheumatic disease.

Chemicals: A definitive linkage between benzene and aplastic anemia has been established from clinical and epidemiologic data, as well as from animal and in vitro studies (Shahidi, 1988, Snyder, 2000). Despite this association, benzene is still widely used as a solvent and in the manufacture of other chemicals, drugs, dyes, explosives, leather goods, and rubber. Chemicals used in insecticides (chlorophenothane), glue (toluene), and Stoddard solvent (petroleum distillates) have also been associated with aplastic anemia.

Drugs: Chloramphenicol was, at one point, the most common cause of drug-induced aplastic anemia in the United States. Anticonvulsant medications, in particular carbamazepine and hydantoins, are also associated with the development of aplastic anemia. The toxic metabolic
intermediate of carbamazepine has been implicated in fatal cases of aplastic anemia (Gerson et al., 1983). Treatment with antineoplastic cytotoxic agents carries a high risk of aplastic anemia, and drugs such as gold salts, D-penicillamine, phenylbutazone, quinacrine, and acetazolamide have also been implicated. Commonly used drugs such as penicillin, furosemide, allopurinol, and nonsteroidal anti-inflammatory drugs (NSAIDs) are linked to a lesser degree with aplastic anemia.

Infectious agents: Some viral infections, notably infectious mononucleosis caused by Epstein-Barr virus, have been associated with aplastic anemia. Whether anemia results from a direct effect by the virus on the bone marrow or from a host immunologic response is unclear. The association between hepatitis and aplastic anemia is also strong, but anemia does not appear to be related to infection with hepatitis viruses A, B, or C, and may be caused by an unknown virus (Brown et al., 1997). Human parvovirus B19, the virus that causes fifth disease, has been linked with pure red cell aplasia but not with severe aplastic anemia. Although some cases of aplastic anemia have been reported with human immunodeficiency virus (HIV) infections, most patients with HIV infection have a cellular bone marrow, despite varying degrees of peripheral cytopenia.

Radiation: Repeated exposure to low doses of radiation has been associated with aplastic anemia. Single exposure to high doses of radiation (such as after a nuclear explosion) is more likely to lead to leukemia rather than aplastic anemia.

The signs and symptoms of patients presenting with aplastic anemia are typically related to the decrease or absence of peripheral blood cellular components (Kelly et al., 1996). The clinical presentation ranges from insidious to dramatic. Because platelets are depleted early in the process of the disease, dependent petechiae, bruising, gum bleeding, buccal hemorrhage, epistaxis, or retinal hemorrhage may be among the first presentations. Because of anemia, patients may complain of shortness of breath, fatigue, or chest pain. Neutropenia or leukopenia may result in fever, chills, or infections. Hepatosplenomegaly, lymphadenopathy, or bone pain are less common in patients with aplastic anemia, but these findings should alert the physician to other diagnoses, such as infection, leukemia, or lymphoma (Alkhouri and Ericson,1999).

Occupational exposure to benzene is a frequent cause of chronic toxicity, which may result in induction of aplastic anemia and neoplastic processes, including leukemias, as well as breast and lung tumors. Proliferative disorders of the hemopoietic system, which most frequently develop in humans exposed to benzene, include chronic myeloid leukemia, acute myeloid leukemia, lymphoblastic leukemia, malignant lymphoma and multiple myeloma. Development of tumors of the hemopoietic system reflects the damage to bone marrow pluripotential stem cells, which leads to anemia, leukopenia or thrombocytopenia and, then, to fully symptomatic aplastic anemia or myeloid leukemia (Snyder, 2000 and Ray et al., 2007).

Toxicity of benzene to be induced , it first must be metabolized to several metabolites which can accumulate in bone marrow where they are further bioactivated by myeloperoxidases and other heme-protein peroxidases to reactive semiquinones and quinones, which lead to the formation of reactive oxygen species (ROS). ROS include superoxide radical anion, hydroperoxyl radical, hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical. These species are generated by many physiological processes and can affect signal transduction cascades by altering the activities of certain protein kinases and transcription factors (Elsayed, 2015).

This study aimed to present a model for induced aplastic anemia by intoxication with benzene subcutaneously and orally.

MATERIALS AND METHODS

Experimental animals

forty male CD1 mice (*Mus musculus*) weighting 20 – 25 g were purchased from the Egyptian Organization for Serological and Vaccine Production, Egypt, were used as experimental animals throughout the present work. The animals were housed individually in plastic cages and acclimated for 1 week before benzene intoxication. Food and water were offered *ad libitum*. Animals were maintained at 22± 2 °C at normal light/dark cycle.

Benzene was purchased from El-Gomhoria Company, Egypt.

Animal Groups

After an acclimation period for 1 week, animals were classified into four groups, each group consists of ten mice as follow:

1-Control group, received only the ordinary mice diet and water and administered with 2ml/Kg saline subcutaneously (sc) daily along the time of experiment.
2-Sc day after day treatment group with 2ml/Kg of benzene for 15 dose (i.e. for 30 days)
3-Sc daily treatment group with 2ml/Kg of benzene for 15 dose (i.e. for 15 days)
4-Oral daily treatment group with 2ml/Kg of benzene for 15 dose (i.e. for 15 days)

Blood collection

Twenty four hours after the last dose of benzene treatment, animals were anaesthetized by diethyl ether, dissected and blood was collected by heart puncture with syringe (3ml capacity). The required amount of blood was collected in two tubes, one of them contain EDTA anticoagulant for hematological studies, the second contain 0.1 ml sodium citrate solution (3.6%) and 0.9 ml blood was added in this tube for detection of prothrombin time.

Hematological Parameters in Bone Marrow Aspirate

Total bone marrow cell count

Total bone marrow cells were counted according to Lezama et al., (2001). As follows: 1- Remove one femur, clean it from muscles, and cut the epiphyses.
2- Inject 1 ml of isotonic saline solution into the medullary channel and receive the cell suspension in a glass tube.
3- Take 10µl of cell suspension and dilute it with 200µl of Turk's solution.
4- Fill the counting chamber of hematocytometer slide by one drop of diluted bone marrow cell suspension under the cover with smooth flow of fluid.
5- Count bone marrow cells in 4 corners of the large squares (64 large squares), and multiply the total count by 50 to get the bone marrow cell count per µl of cell suspension.

Differential count of bone marrow aspirate

Differential count for bone marrow aspirate was made as follows:
1- Remove the other femur, clean it from muscles, and cut the epiphyses.
2- Insert a needle into one side of the femur and receive bone marrow aspirate from the other side on a clean and dry glass slide.
3- Spread it by using another slide to make bone marrow aspirate film. 4- Dry the film in air.
5- Fix it with absolute methyl alcohol.
6- Place the bone marrow film in diluted Giemsa stain (1:10 with distilled water) (Atlas Medical Company, UK) for 45 minutes.
7- Wash with distilled water and allow to dry.

Observe under oil immersion lens and differentiate bone marrow cell types.

Determination of Hematological Parameters in Peripheral Blood

Hemoglobin in blood was determined according to method of Van Kmpen and Zijlstra (1961) using the kit of Randox Company, United Kingdome.

Red blood cells, white blood cells, platelets, and reticulocytes were counted using hematocytometer method according to Krupp et al. (1976).

PCV percentage was determined according to Turgeon (2005) using microhematocrit tubes coated with anticoagulant.

Leucocyte differential counted was preceded according to Turgeon (2005) by using Giemsa stain (Atlas Medical Company, UK).

Prothrombin time detection was preceded according to Turgeon (2005). This basic procedure involves adding plasma on an excess of extrinsic thrmboplastin-Ca substrate by using thrmboplastin-Ca kit (Biomeriux – France).

Statistical Analysis

Data are expressed as mean±SD. The level of statistical significance was taken at $P < 0.05$, using one way analysis of variance (ANOVA) test followed by Dunnett test to detect the significance of differences between each group and control. All analysis and graphics were performed by using, INSTAT and graphPad Prism software version 4.

RESULTS

The results were tabulated in three tables and nine figures. The results of intoxicated CD1 mice with benzene by different routes of administration were compared with control.

Hematological parameters in peripheral blood

Hematological parameters were examined in blood samples obtained by heart puncture and mixed with anticoagulant (EDTA). These samples were analyzed for red blood cells (RBCs), white blood cells (WBCs), and platelet count also for determination of hemoglobin concentration (Hb), hematocrit, reticulocytes percentage and differential count for WBCs.

The influences of benzene intoxication (15 dose 2ml/Kg Sc. day after day) as illustrated in figures (2-4) showed that, intoxication with benzene resulted in a state of decline in hemoglobin concentration (-11.8%), RBCs count (-37%) and hematocrit (-15.7%) significantly compared to control ($P<0.05$). Also in the animal group, treated orally (daily with 2ml/Kg of benzene), showed decrease in Hb, RBCs and hematocrit significantly compared to control ($P<0.05$) with percentage of differences,-23.25, -26.58 and -14.4 respectively. on the other hand, the animals which treated daily for 15 day (2ml/Kg of benzene Sc.),did not show any significant change in Hb and hematocrit, but RBCs count decline only with -23%

Blood indices (MCH, MCHC and MCV) were examined in this study and MCH was significantly declined in all treated groups (-33.6%, -24.6 and -39.1) compared to control, but MCV did not affect by benzene intoxication in all groups. MCHC was significantly reduced only in the fourth group which treated with benzene orally (daily) by -28.7 as illustrated in table 2.

Table-1: Differential leucocytes count as affected by intoxication of male mice with benzene

Animal groups	Control (Mean±SD)	Sc day after day treatment (Mean±SD)	Sc daily treatment (Mean±SD)	Oral daily treatment (Mean±SD)
Neutrophils%	25.86 ±4.26	38.79 ±4.569 **	33.70 ±3.994 *	34.00 ±6.633 *
Lymphocytes%	72.17±4.167	53.65 ±4.904 **	59.00±3.606 **	58.67±7.63 **
Monocytes%	4.976 ±0.6301	5.458 ±1.422	4.500 ±0.9354	4.125 ±1.315
Eosinophils%	1.453 ±0.2979	1.492 ±0.3005	1.500 ±0.3536	1.260 ±0.3362
Basophils%	0.8050 ±0.05447	0.7775 ±0.1167	0.6750 ±0.2363	0.5400 ±0.2881

(*) significant difference compared to control group ($P < 0.05$).
(**) highly significant difference compared to control group ($P < 0.01$).

Table-2:Blood indices as affected by intoxication of male mice with benzene

Animal groups	Control (Mean±SD)	Sc day after day treatment (Mean±SD)	Sc daily treatment (Mean±SD)	Oral daily treatment (Mean±SD)
MCH (pg/cell)	27.47 ±5.333	18.32 ±4.997 **	20.70 ±2.926 *	16.73 ±2.575 **
MCHC%	31.70 ±2.938	32.16 ±2.038	31.94 ±3.152	22.58 ±11.02 *
MCV (fl/cell)	64.65 ±16.68	60.16 ±11.88	63.25 ±12.26	57.80 ±6.979

(*) significant difference compared to control group ($P < 0.05$).
(**) highly significant difference compared to control group ($P < 0.01$).

Reticulocytes are erythrocytes still possessing RNA. The enumeration of reticulocytes is important in assessing the status of erythrocyte production in the bone marrow. By observing figure (7) was noticed that a huge depression in reticulocyte percentage by intoxication with benzene (Sc and oral daily treatment) to reach about the third of control in orally treated group (-71.6%) and -48.1 in Sc daily treated group ($P<0.01$), on the other hand, the animal group which treated subcutaneously with 2ml/Kg of benzene day after day, did not show significant change in this parameter.

A huge decline in total WBCs count as a marker of benzene hematotoxicity as illustrated in figure (5) in all intoxicated groups with benzene to reach about the half of control, this decrease mainly in lymphocytes number (table 1). Depression occurred also for platelets in all intoxicated groups with benzene by Sc daily, Sc day after day or oral daily treatment (2ml/Kg) as follow, -51.8, -56.6 and -31.3 respectively compared to control ($P<0.01$).

Prothrombin time is prolonged by benzene intoxication subcutaneously (day after day) which means that the activity of prothrombin was reduced. The time is increased by 14.62% compared to control (figure 8) ($P<0.05$), on the other, no significant change in the other intoxicated groups.

Hematological parameters in bone marrow aspirate

The data obtained from bone marrow aspirate examination were illustrated in figures (1) and table (3), these to give us a complete picture about the bad effects of benzene intoxication on bone marrow cellularity, maturation of bone marrow cells to give the blood cells and percentages of myeloid and erythroid precursor cells.
By making total count for bone marrow nucleated cells, observed sever bone marrow depression caused by benzene in all intoxicated groups with benzene by Sc daily, Sc day after day or oral daily treatment (2ml/Kg), as follow, -40.57%, -54.2% and -32% respectively ($P<0.01$).

By studying the myeloid precursor cells, differential count for the stained bone marrow aspirate films, a slowing in a maturation rate of cells by benzene intoxication was occurred as illustrated by table (3) , they showed a shift toward immature cells, in other mean, the immature cell percentages were elevated compared to control and mature cells percentages were decreased by a remarkable degrees.

promyelocytes percentages did not affected in all intoxicated groups with a remarkable degrees. On the other hand, by measuring the percentages of myelocytes in the three treated groups we detected that, there is a significant increase in this parameter in subcutaneously treated day after day, daily, and orally treated daily groups by 64.4%, 36.3% and 41.5% respectively. A significant depression of band granulocyte percentages occurred as a result of benzene intoxication with about a third of control in day after day treated group subcutaneously (-34.6%) ($P<0.01$) and by -19.26 in orally treated group ($P<0.05$).

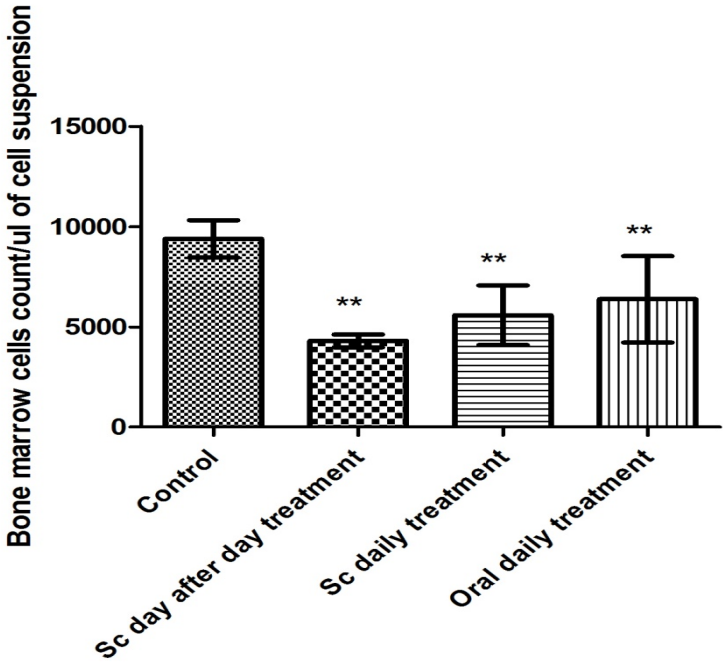

Figure (1) Bone marrow cells count as affected by intoxication of male mice with benzene
(*) significant difference compared to control group ($P < 0.05$).
(**) highly significant difference compared to control group ($P < 0.01$).

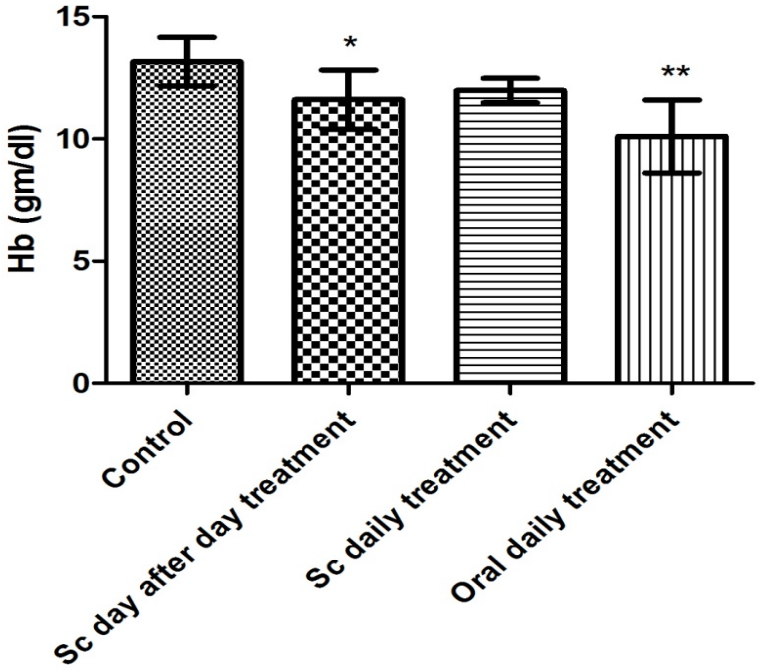

Figure-2: Hemoglobin concentration as affected by intoxication of male mice with benzene

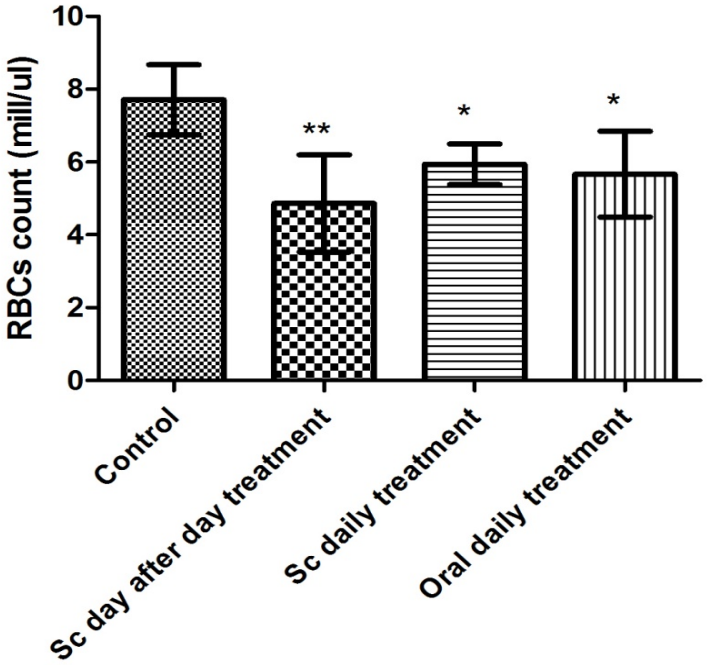

Figure-3: Red blood cells count as affected by intoxication of male mice with benzene

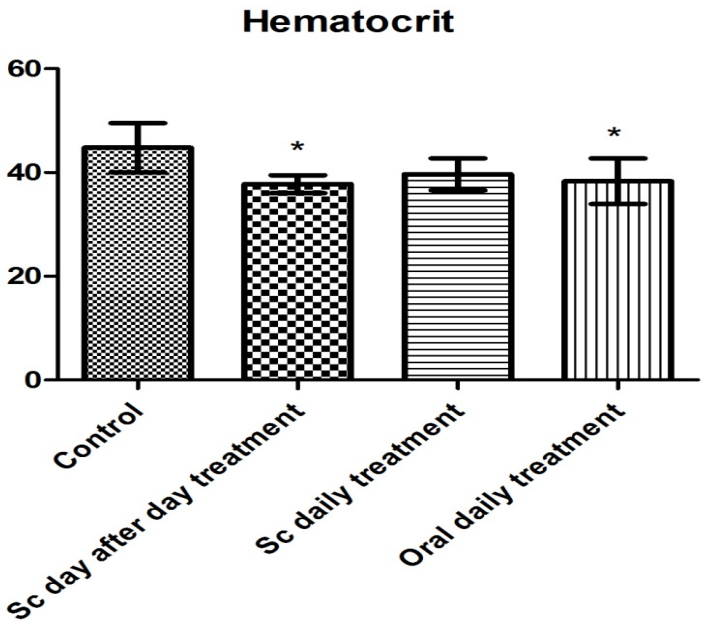

Figure-4: Hematocrit as affected by intoxication of male mice with benzene

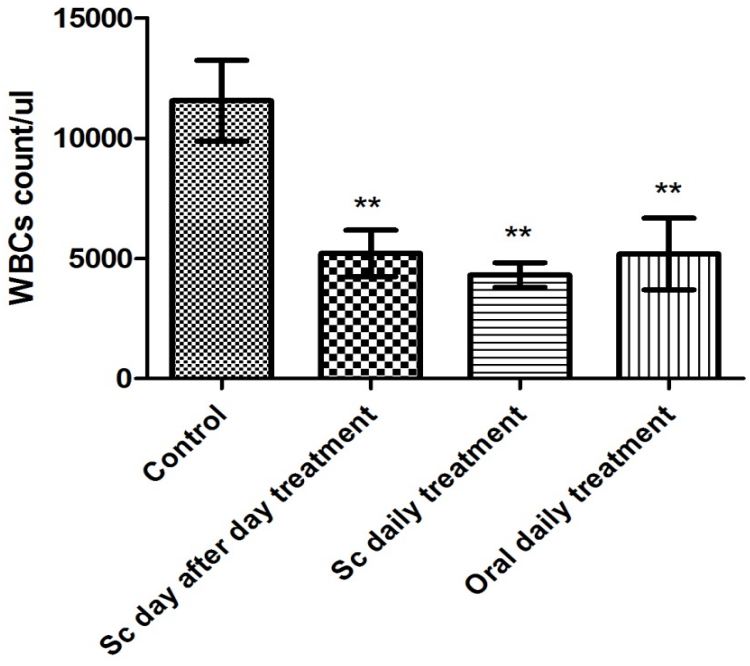

Figure-5: White blood cells count as affected by intoxication of male mice with benzene

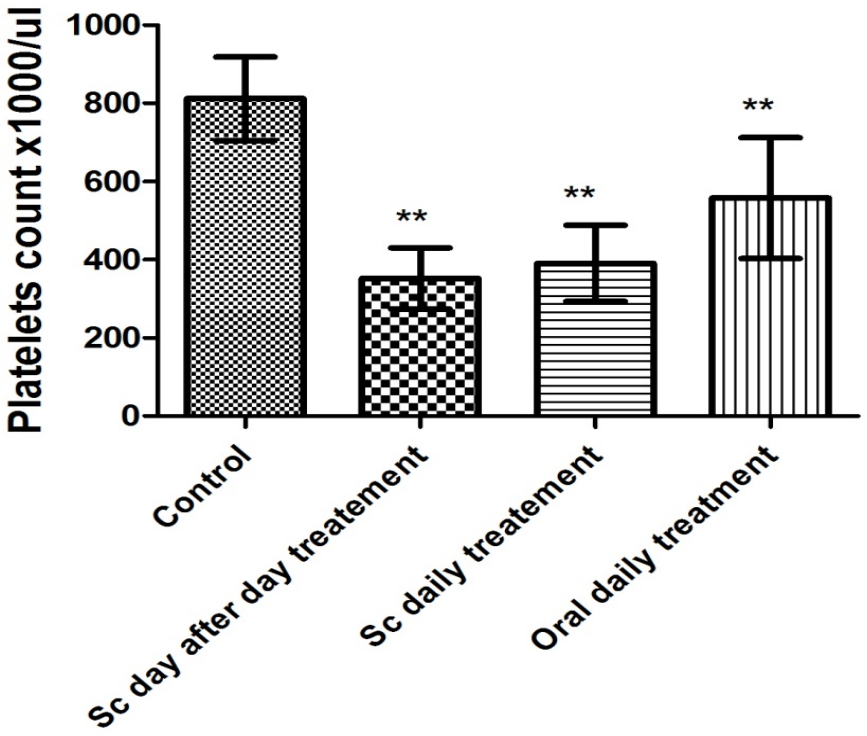

Figure-6: Platelets count as affected by intoxication of male mice with benzene

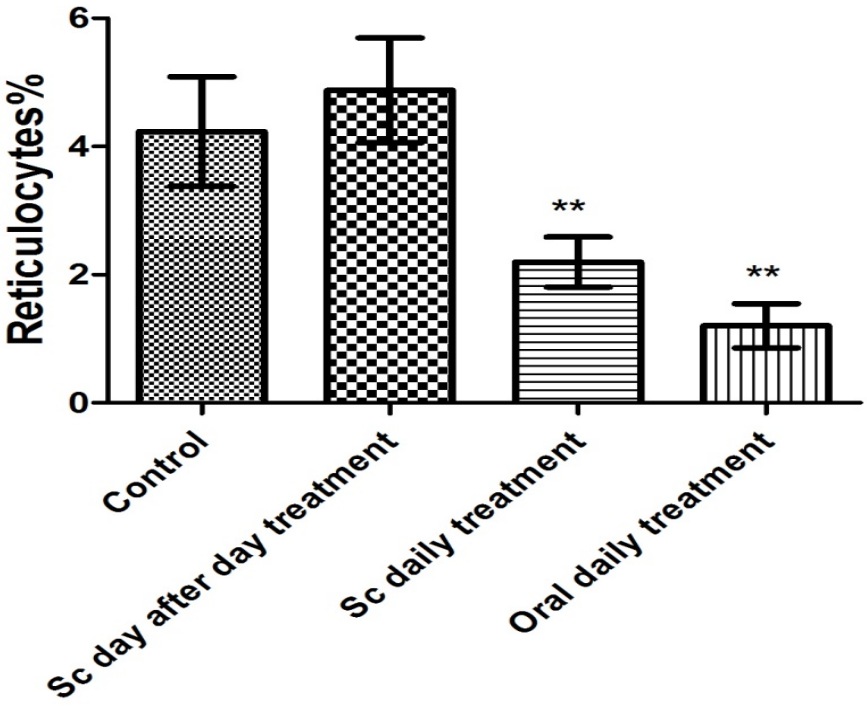

Figure-7: Reticulocytes count as affected by intoxication of male mice with benzene

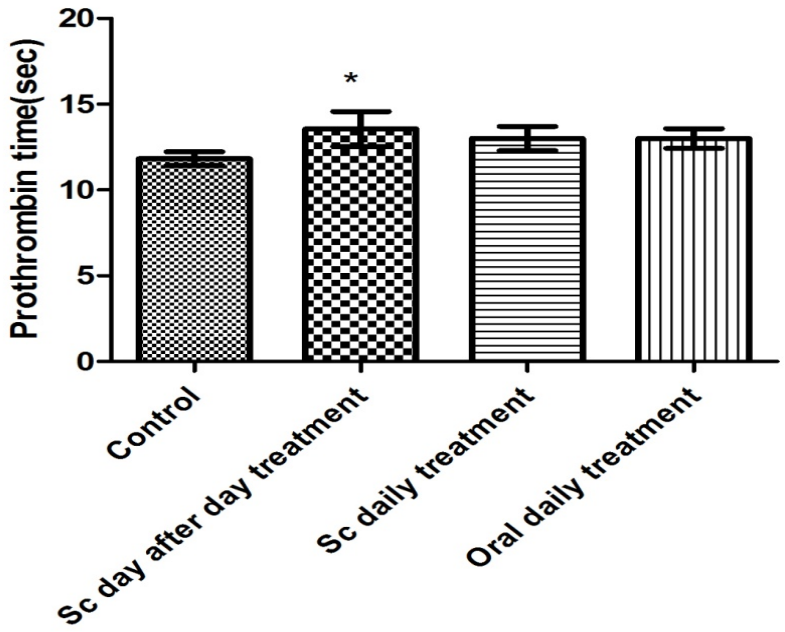

Figure-8: Prothrombin time as affected by intoxication of male mice with benzene

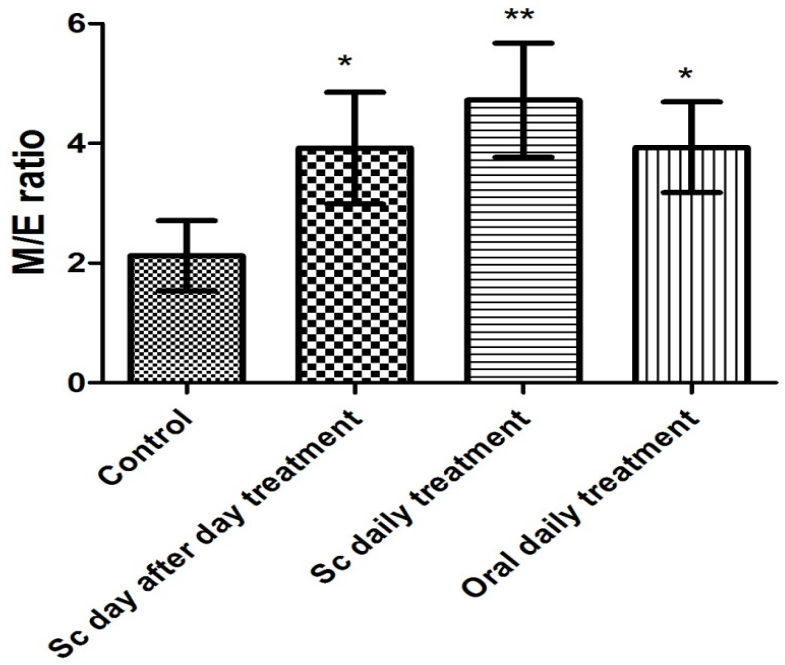

Figure-9: Myeloid/Erythroid ratio as affected by intoxication of male mice with benzene
(*) significant difference compared to control group ($P < 0.05$).
(**) highly significant difference compared to control group ($P < 0.01$).

The rate of maturation of erythroid precursor cells also became slower by benzene intoxication, the percentages of cells were shifted toward the immature cells to yield a case of hypocellularity (table 3). Proerythroblasts showed significant decrease by benzene treatment in all croups compared to control ($P<0.01$).

Basophilic erythroblasts did not show any significant changes in all treated groups and polychromatic erythroblasts showed only in the group which treated day after day subcutaneously with benzene a remarkable decrease (-61%) when compared to control (P<0.01), On the other hand a significant depression occurred to orthochromatic erythroblasts percentage in all groups intoxicated by benzene compared to control (P<0.01)

Table-3: Differential bone marrow cells count as affected by intoxication of male mice with benzene

Animal groups	Control (Mean±SD)	Sc day after day treatment (Mean±SD)	Sc daily treatment (Mean±SD)	Oral daily treatment (Mean±SD)
Myeloblast%	0.9750 ±0.1012	0.4275 ±0.1896 **	0.2960±0.0698 **	0.4320 ±0.127 **
Promyelocytes%	0.6120 ±0.1964	0.3680 ±0.09011	0.5920 ±0.1397	0.7780 ±0.1641
Myelocytes%	13.50 ±2.235	22.20 ±1.558 **	18.40 ±1.639 *	19.10 ±3.190 **
Metamyelocytes%	14.61±1.830	15.75 ±2.304	15.90 ±2.724	14.29 ±2.916
Band granulocytes%	34.68 ±1.863	22.67 ±1.528 **	31.25 ±1.500	28.00 ±4.830 *
Proerythroblasts%	0.8200 ±0.1549	0.4300 ±0.1892 **	0.4175 ±0.086 **	0.4840 ±0.085 **
Basophilic erythroblasts%	0.980 ±0.0735	1.60 ±0.300 *	1.80 ±0.173 **	1.11±0.349
Polychromatophilic erythroblasts%	4.329 ±1.153	1.688 ±0.2650 **	4.167 ±1.422	4.250 ±1.258
Orthochromatophilic erythroblasts%	21.20 ±4.817	11.72 ±3.244 **	11.63 ±2.981 **	11.04 ±3.968 **

(*) significant difference compared to control group (P < 0.05).
(**) highly significant difference compared to control group (P < 0.01).

DISCUSSION

The mechanism of benzene hematotoxicity is not clear yet. One theory stresses the importance of active benzene metabolites and cellular DNA adducts, particularly inherited in somatic cell lines, which cause inability of the cells to react to cytokines, resulting in excessive proliferation. Chromosomal aberrations induced by binding active benzene metabolites to DNA may also lead to oncogenes activation or antioncogenes inactivation (Stoyanovsky et al., 1995, Xie et al.,2005, Barreto et al.,2009). Another hypothesis is associated with stimulatory functions of the bone marrow microenvironment. Cytotoxic damage of bone marrow stromal cells and macrophages, in particular induced by benzene and its metabolites, affects their capacity to control proliferation and differentiation of stem cells. Failure to synthesize normal signaling polypeptides results in uncontrolled proliferation of cells which would otherwise be inhibited or the cells would be forced to differentiate into individual cell lines. In a similar way, a disturbed immune system including lymphocytes and stromal macrophages may also lead to development of neoplastic cells, which in physiological conditions would be detected and selected out by the immune surveillance mechanisms (Post et al., 1986, King et al.,1988, Renz et al.,1991, Bogadi-Sare et al.,2000, Lee et al.,2010). Hematotoxic activity of benzene involves both, the solvent and its metabolites. Induction of the cytochrome P-450 system, especially CYP 2E1 and CYP 2B1, which participates in metabolic activation of benzene, may lead to intensification of the observed alterations (Snyder et al., 2000).

The present data showed that subcutaneous and oral intoxication with benzene induced neutrophilia in male CD1 mice. This probably caused due to intensive mobilization of segmented cells from the bone marrow compartment (Macedo et al., 2006). The relative increase in neutrophils may also have been due to a non-specific response, or to a response to the specific stimulus of benzene metabolites, such as hydroquinone, which stimulate the granulocyte/macrophage progenitor cells both in vivo and in vitro (Lezama et al., 2001; Macedo et al., 2006).

There is a characteristic signs of aplastic anemia as decreased bone marrow cell counts associated with decreased peripheral erythrocyte, platelet, and leukocyte counts in male CD1 mice. The present results are in accordance with other authors who have injected mice with benzene subcutaneously (Lezama et al., 2001) also the study of Irons et al. (2005) who discovered bone marrow displasia in 23 workers exposed to high concentrations of benzene, this benzene-induced displasia include marked dyserythropoeisis, eosinophilic dysplasia and abnormal cytoplasmic granulation of neutrophilic precursors. Hematophagocytosis, stromal degeneration and bone marrow hypoplasia also seen in the study of irons et al. (2005).Also the study of Liu et al. (2001) on mice inhaled benzene and developed a type of aplastic anemia.

The bone marrow is an ordered environment with the hemopoietic stem cell being in close proximity to protective stromal cells. Ordered maturation of myeloid progenitors can be seen in relation to the normal hemopoietic stem cells and one of the diagnostic features of myelodysplasia is abnormal location of these immature precursor cells. In addition to these myeloid components, mature B and T cells are present which may exert significant effects on the stem cell compartment. Thus, hemopoietic stem cells are found in a relatively protected environment within the bone marrow. Oxygen and toxins are delivered by the vascular system, and the differentiating myeloid precursors, which are rich in myeloperoxidase, provide an environment which easily generates oxidative stress (Morgan and Alvares, 2005).

Various benzene metabolites can cause oxidative DNA damage, lipid peroxidation in vivo, formation of hydroxylated deoxyguanosine residues in DNA, and DNA strand breaks, thus, implicating a role for reactive oxygen species (ROS) in benzene-induced toxicity (Faiola et al., 2004). Formation of DNA double-strand breaks by ROS and other mechanisms can lead to increased mitotic recombination, chromosomal translocations, and aneuploidy. Such genetic consequences may result in protooncogene activation, tumor suppressor gene inactivation, gene fusions, and other deleterious changes in stem cells that can ultimately result in leukemic responses. Thus, DNA damage following benzene exposure must be properly repaired or the affected cells must be eliminated to prevent proliferation of mutated cells and subsequent transformation into malignancies (Faiola et al., 2004).

Various studies have suggested that hematopoietic stem cells (HSC) are the target cells for benzene-induced alterations. In the bone marrow (BM), HSC are a small population (<0.05% of BM cells) of self-renewing, pluripotent cells that give rise to all blood cells (Morrisson et al., 1994). Inhalation exposure to benzene significantly reduced the number of transplantable spleen colony-forming units (CFU-S), granulocyte/monocyte colony-forming units (CFU-GM), and erythroid colony-forming units (CFU-E) in the bone marrow of male and female mice, indicating a decrease in the number of HSC following exposure to benzene. Benzene was found to affect cell cycle kinetics as the fraction of CFU-GM in S phase was suppressed in male mice exposed to 300 ppm benzene for 2 weeks compared to unexposed control mice (Yoon et al., 2001). In addition, persistent benzene-induced DNA damage was observed as an increased frequency of aneuploidy in the long-term self-renewing population of HSC (Lin−, c-kit+, Sca-1+) from male and female mice 8 months after gavage with benzene compared to the corn-oil-exposed control mice. Thus, benzene has short- and long-term deleterious effects on HSC (Giver et al., 2001).

Exposure to 100 ppm benzene for 2 weeks was sufficient to cause hematotoxicity in the blood and bone marrow as well as significant genotoxicity. At the time point examined, the percentage of dead cells and cells undergoing apoptosis in the bone marrow did not change in response to inhaled benzene. The benzene metabolite hydroquinone can inhibit apoptosis, and some have postulated that damaged cells that have not been repaired could then proliferate and thereby lead to leukemia (Snyder, 2000). Other investigators found that treatment of HL60 human promyelocytic leukemia cells and CD34+ human bone marrow progenitor cells with some benzene metabolites induced time- and concentration-dependent apoptosis (Moran et al., 1996).

Martınez-Velazquez et al. (2006) found that HepG2 cells exposed to 75 mM of benzene produced mainly muconic acid (an indicator of muconaldehyde formation). The benzene and metabolite combination was able to induce a more marked viability decrease than benzene alone. They explored the possible induction of apoptosis as the cause of this viability decrease. They also found morphological and biochemical changes typical of this type of cell death.

Intoxication of mice with benzene severely reduces the number of hemopoietic and lymphoid cells in the peripheral blood by inhibiting DNA synthesis in progenitor cells in the bone marrow. Studies by Cronkite et al. (1982) and Farris et al. (1997) indicated that the cycling fraction of hemopoietic stem cells is elevated dramatically by benzene, whereas a study by Lee et al. (1988) indicated that benzene suppresses these parameters.

Lymphopenia, a decreased number of lymphocytes in peripheral blood is the most common disorder resulted from benzene exposure in many epidemiological studies (Irons et al.,2005; Tsai et al., 2006) and in animal studies (Lezama et al., 2001; Macedo et al., 2006). These results in accordance with the present results which showed a remarkable decrease in peripheral lymphocytes .

The hemopoietic system dynamically but conditionally responds *in vitro* as well as *in vivo* to the various endogenous and exogenous stimuli that induce changes in blood and hemopoiesis in normal mice under highly controlled conditions (Yoon et al., 2001).

The sensitivity of persons to benzene toxicity elevates with possessing high levels of CYP2E1 and myeloperoxidase, and low levels of GSH transeferase and NQ01. On the other hand the resistance to benzene toxicity increases with low levels of CYP2E1 and myeloperoxidase, and high levels of GSH transferase and NQ01.

Snyder (2000; 2002 and 2004) reported that progressive bone marrow depression ranging from leucopenia, anemia, or thrombocytopenia, through pancytopenia indicative of bone marrow aplasia. In addition recognizing that aplastic anemia was one end point in benzene toxicity and acute leukemia, primarily of myelocytic variety, is an alternative end point to benzene toxicity.

Benzene toxicity can be characterized with respect to the intensity and duration of exposure. Chronic exposure to low doses over a period of weeks or months may lead to decrease in circulating blood cells, and, to some extent in stem cells, but sufficient functional residual capacity of the bone marrow may remain to permit circulating cell levels to be restored to normal if exposure to benzene ceases. Exposure to high levels of benzene may cause sufficient loss of both stem cells and cells of bone marrow stroma that pancytopenia and aplastic anemia results. At intermediate exposure the bone marrow may appear dysplastic, a condition characterized by abnormal morphology, inadequate hematopoiesis, and chromosome damage (Snyder, 2000).

Myelodysplastic syndrome in considered to be preleukemic state and usually proceeds to give rise to full blown leukemia. It is significant, that damage to the immune system can lead to death in aplastic anemia, myelodysplastic syndrome or acute myelocytic leukemia (Snyder, 2000). This in agreement with the present results in that benzene inhalation caused a high degree of hypocellularity in bone marrow appeared with bone marrow aspirate and bone marrow biopsy examinations, also a marked decrease in leukocyte, erythrocyte and platelet counts in peripheral blood.

Hazel and Kalf (1996), using 32D cells, reported that hydroquinone promoted cellular proliferation and differentiation of the myeloblast to the myelocyte stage, but inhibited further maturation to the neutrophil. Normally the size of the myelocyte pool is controlled by the rate of myelocyte formation and the rate of maturation to neutrophils. When the numbers of myelocytes exceed the need, some cells undergo apoptosis. In the present study was noticed shifting toward immature leukocytes especially myelocytes and metamyelocytes on the other hand decrease of the mature band granulocytes which means slow rate of maturation of leukocytes or inhibiting it by benzene intoxication, also erythropoisis became slower by benzene intoxication which appeared by increasing the percentages of immature erythroblast (proerythroblasts, basophilic erythroblasts, and polychromatic erythroblasts) and decrease the more mature orthrochromatic erythroblasts, and reticulocytes percentage also decreased in peripheral blood, this inhibiting effect of benzene for leukocytes and erythrocytes maturation discuss the low count of total leukocytes and erythrocytes in the present results, and these results in accordance with the previous discussion of Hazel and Kalf.

Hazel et al. (1996) demonstrated that hydroquinone can also inhibit apoptosis thereby resulting in expansion of the clone of myelocytes. Any mutated cells in this population, which have not undergone DNA repair, will now proliferate, and in effect, promote the development of the leukemia. It may be that the example of benzene-induced promotion was observed in a study of Spalding et al. (1999).

Among the hematological alterations reported to be associated with benzene exposure, peripheral lymphocytes of benzene-exposed workers have shown higher frequencies of somatic mutations and several types of chromosomal aberrations and aplastic anemia, which is characterized by pancytopenia, variable hypocellularity of the bone marrow in the absence of a malignant myeloproliferative disease. Aplastic anemia may be caused by anomalies at three different stem cell functional levels, (A) Bone marrow microenvironment abnormalities, which modify totipotential cell differentiation; (B) inadequate function of cellular hematopoietic regulators (T lymphocytes and their lymphokines); and (C) Immunological inhibition of hematopoiesis (Lezama et al., 2001).

Thrombocytopenia which appeared in the present study as a result of bone marrow depression reflected only in platelet count and increase in prothrombin time which means decrease in prothrombin concentration. On the other hand megakaryblast and megakaryocyte

percentages did not show any significant changes, this may be due to their low percentages, and thus the statistical analysis could not determine the significance of differences. These results are in accordance with Escorcia et al. (1997) study on rats administered benzene orally and bleeding from nasal and gastric mucosa occurred as a result of low platelets count and prothrombin concentration.

CONCLUSION

The study concluded that, by benzene intoxication, hematological parameters in peripheral blood and bone marrow was affected as follow: 1-Reduction in blood cell counts was occurred, in RBCs, WBCs, platelets, and hemoglobin. Lymphocytes percentages in blood were depressed and neutrophils percentages were elevated in all intoxicated groups. 2- Bone marrow depression was occurred by benzene as a reduction in bone marrow cellularity and slow rate of cells maturation.

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ISSN : 0976-4550

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