

# In vitro and in vivo evaluation of toxic effect of benzene on lymphocytes and hepatocytes.

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## Citation

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## Abstract

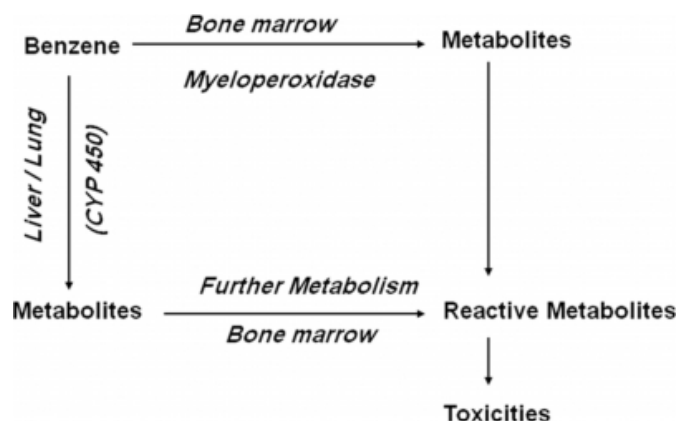
**Objective:** Explore the hepato and hematotoxicity of benzene through in vitro and in vivo evaluation. **Methods:** Splenic lymphocytes and hepatocytes were used for the in vitro study. Liver enzymology and lymphocytes were evaluated in 154 petrol filling workers with <10 years and >10 years of exposure and 33 referents. **Results:** Lymphocytopenia was most marked in study group than in controls. Absolute lymphocyte count were reduced to  $1.67 \pm 0.3 \times 10^9/\text{mL}$  and  $1.49 \pm 0.4 \times 10^9/\text{mL}$  in workers with <10 years and >10 years of exposure than the control ( $1.9 \pm 0.3 \times 10^9/\text{mL}$ ). This was supported by our in vitro result showing a negative correlation between reduction in viability of lymphocytes and increasing concentration of benzene. The mean value of ALP and ALT in study group was significantly lowered than in control group. The total protein level increased within the normal range in study group than in controls whereas albumin level decreased in study group in contrast with control group. Mean  $\pm$  SD values of AST and total bilirubin in study group were not elevated from the corresponding value in control group. **Conclusion:** These in vitro and in vivo results show that human lymphocytes and hepatocytes are sensitive targets of benzene.

## INTRODUCTION

Benzene is a ubiquitous industrial solvent and widely distributed environmental contaminant that has been linked to adverse health effect in humans and animals [1-3]. Benzene ranks as the 17th chemical in terms of total annual production in 1994 [4] and therefore represents a significant occupational hazard. Hematotoxicity is the most noted and characteristic systemic effect resulting from intermediate and chronic benzene exposure leading to aplastic anemia, leucopenia and thrombocytopenia [5,6]. Other health effects of benzene include immunological changes which appear largely related to decrease in circulating leukocytes and the ability of lymphoid tissue to produce mature lymphocytes necessary to form antibodies [7,8]. Chronic exposure results in consistent structural and numerical chromosomal aberrations in lymphocytes and bone marrow cells. Benzene metabolites covalently bind to cellular macromolecules (including DNA, RNA and proteins) thereby leading to dysfunction in the bone marrow and other tissues. Along with benzene, one or more reactive benzene metabolites are involved in the health effects. Majority of benzene metabolism occurs in liver by cytochrome P450 and a small amount is metabolized in the bone marrow [9-11], the site of characteristic benzene toxicity (Figure 1).

## Figure 1

Figure 1: Shows the diagrammatic representation of benzene metabolism in Liver and Lungs and further production of reactive metabolites in bone marrow



Benzene and its metabolites induce cell transformation, gene mutation and apoptosis in mammalian cells in culture [12]. Liver function as the key organ of metabolism and excretion of benzene thus, constantly endowed with the task of detoxification, inducing various disorders to the organ. Cultured mammalian hepatocytes retaining differentiated hepatic functions would be great useful in toxicology [13,14]. Since identification of morphological and structural changes induced by benzene and its metabolites to the

hepatocytes in living animals is a great challenge. Changes in the hepatic function induced by these metabolites in humans can be correlated with the changes in liver function test (LFT) [15,16]. Therefore, we conducted this study to evaluate the toxic effect of benzene by in vitro and in vivo methods. Cultured lymphocytes and hepatocytes were used as in vitro model systems and the results were correlated with the lymphocyte count and the enzymology of liver in petrol attendants who were exposed to benzene.

## **MATERIALS AND METHODS**

### **CHEMICALS**

Acetonitrile, Dimethyl Sulphoxide, Hepatocyte Incubation media and benzene were from Sigma, St Louis, MO, USA. Fetal calf serum purchased from Gemini Bio-Products, Inc., Calabasas, CA, USA. All other reagents used were analytical grade.

### **ANALYSIS OF BENZENE TOXICITY**

#### **VIABILITY OF LYMPHOCYTE AND BENZENE EXPOSURE**

Spleen cells were isolated from C57BL/6 mice as described earlier [17], FACS caliber analysis has done to conform the purity of the spleen cells and found 100% pure (Data not shown). Eight hours after incubation (5% of CO<sub>2</sub> at 37oC) with different concentration of benzene (1ppm, 2ppm, 3ppm, 4ppm, 5ppm, 7ppm and 10ppm), viability of splenic lymphocytes (5 x 10<sup>6</sup> cells/ ml) was estimated by the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] dye conversion assay. Results were expressed as mean percentage viability ± SD for four replicates.

#### **VIABILITY OF HEPATOCYTES AND BENZENE EXPOSURE**

Parenchymal component of liver cells was isolated one day prior to the study and seeded in 24 well plates in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum (FCS) and Pencillin as described elsewhere [13]. After 2 hours the medium was replaced to remove floating cells. Eight hours after incubation cell viability was estimated with different concentrations of benzene (1ppm, 2ppm, 3ppm, 5ppm, 7ppm, and 10ppm) by MTT assay.

### **ANALYSIS OF BENZENE TOXICITY**

#### **STUDY POPULATION**

The study was conducted on 154 male benzene exposed petrol filling workers (group I: <10 years of exposure, group

II: >10 years) and 33 healthy control subjects were matched for demographic properties with the study group (Table1). Smokers and subjects suffering from any chronic illness were excluded from the study. Written informed consent was obtained from each subject prior to the study. Venous blood sample (3mL) was obtained in heparinised tubes from workers and controls. The study was approved by the Institutional Ethics Committee of Deccan College of Medical Sciences, Hyderabad.

### **Figure 2**

Table 1: Shows the demographic details of the subjects, in bracket details of the controls included. (Smokers were excluded from the study)

Group	Age	Number of Subjects
Group I <10 years of exposure (Control I)	28±11 (26±13)	94 (19)
Group II >10years of exposure (Control II)	36±14 (33±16)	60 (13)

#### **BLOOD ANALYSIS FOR TOTAL LYMPHOCYTE AND LIVER FUNCTION**

Blood samples were analyzed by a Coulter TS40 blood counter which was calibrated daily. Approximately 10<sup>4</sup> white blood cells were counted to provide a total WBC count and a lymphocyte percentage, which are multiplied together to generate the absolute lymphocyte count. Differential leukocyte count was done according the method given elsewhere [18]. All abnormal counts were repeated manually. Serum concentrations of total protein, albumin, total bilirubin, ALT, AST and ALP were determined using Beckman's autoanalyser.

### **STATISTICAL ANALYSIS**

All the data was expressed as Mean ± Standard deviation. Statistical comparison between different groups were performed using one-way analysis of variance (ANOVA) and tukey test for average comparison, significance was accepted at p < 0.05. Statistical analysis was performed using ORIGIN version 6 and SPSS version 10.0.

## **RESULTS**

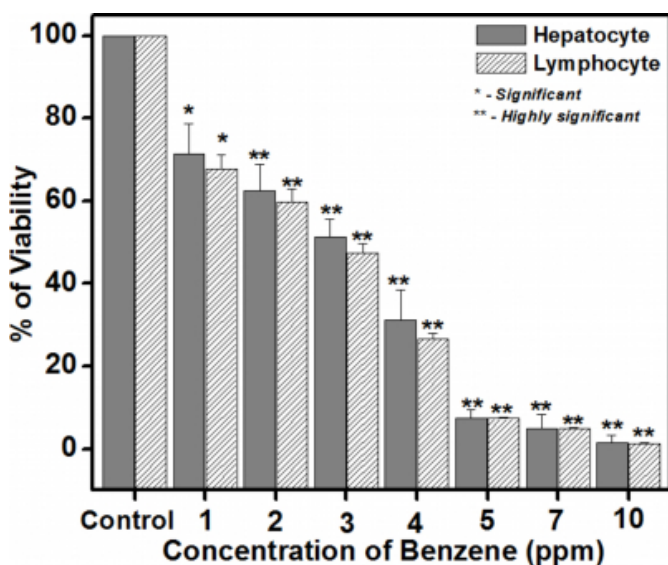
### **ANALYSIS OF BENZENE TOXICITY**

To examine the lymphocyte and hepatocyte toxicity of benzene and its metabolites, we performed in vitro analysis using cultured spleen cells and hepatocyte in the absence and presence of various concentrations of benzene under conditions that favored proliferation and differentiation along the cell replication pathways. Figure 2 shows the

viability of cultured spleen cells with different concentrations of benzene (1ppm, 2ppm, 3ppm, 4ppm, 5ppm, 7ppm, and 10ppm). Significant lymphotoxicity was observed ( $p=0.000$ ) after exposure to various concentrations of benzene. There was a significant reduction in the lymphocyte viability (67%) at 1ppm concentration of benzene and with further increase in the concentration of benzene to 2ppm, 3ppm, 4ppm, 5ppm, 7ppm and 10ppm, the viability was decreased to 59%, 47%, 26%, 7%, 5% and 1% respectively. Figure 2 also shows the viability of hepatic cells cultured for 8 hours with different concentrations of benzene. With 1ppm concentration of benzene, 30% decrease in the viability was observed and as concentration of benzene further increased to 2ppm, 3ppm and 4ppm, 40%, 50% and 70% decrease in viability was observed. These in vitro results were confirming that benzene is toxic to both lymphocytes and hepatocyte.

**Figure 3**

Figure 2: Shows the effect of different concentrations of benzene on the viability of cultured hepatocytes and freshly isolated spleen lymphocytes after 8hrs of incubation. Results are represented as mean  $\pm$  SD,  $n=4$ .



**ANALYSIS OF BENZENE TOXICITY**

Figure 3 shows the benzene toxicity to Lymphocytes in petrol attendants. Percentage of lymphocyte count in control subjects was found to be  $37.92 \pm 1.89$  whereas a border line significant decrease ( $32.3 \pm 1.65$ ,  $p=0.056$ ) was observed in subjects exposed to benzene for less than 10 years (Almost 15% reduction in lymphocytes). However, lymphocyte count further significantly decreased to  $29.3 \pm 1.76$  ( $p=0.03$ , almost 23% reduction in lymphocytes) in workers with more than 10 years of exposure. A significant reduction in absolute

lymphocyte count was observed in study groups ( $1.67 \pm 0.3 \times 10^3/\mu\text{L}$  for  $<10$  years (13% reduction) and  $1.49 \pm 0.4 \times 10^3/\mu\text{L}$  for  $>10$  years (22% reduction) compared to controls ( $1.9 \pm 0.3 \times 10^3/\mu\text{L}$ ).

**Figure 4**

Figure 3: Bar diagram shows the relation between the years of exposure to benzene and percentage of lymphocyte count in blood of petrol attendants. Inset shows the absolute lymphocyte count. Results are represented as mean  $\pm$  SD.

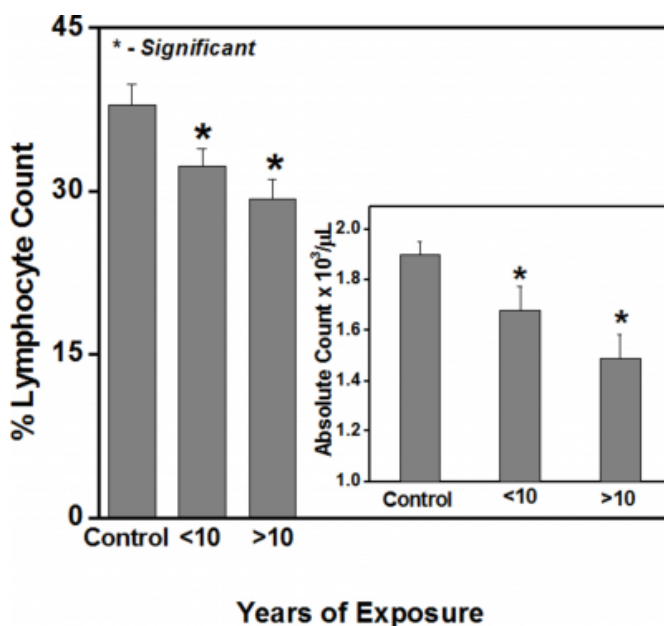


Table 2 shows the mean  $\pm$  SD levels of total protein, albumin, ALT, AST, ALP and total bilirubin in petrol attendants with corresponding value in controls. The mean value of ALP ( $135 \pm 23$ ) and ALT ( $41 \pm 11$ ) in study group was significantly lowered ( $p=0.03$  and  $p=0.04$  respectively) than the corresponding values in the control group ( $165 \pm 34$  and  $63 \pm 13$  respectively). The total protein level increased within the normal range in exposed group ( $7.98 \pm 2.86$ ) than the control group ( $7.31 \pm 3.23$ ) whereas albumin level decreased to  $5.10 \pm 0.32$  in exposed group in comparison with control group ( $5.25 \pm 0.21$ ). Mean  $\pm$  SD values of aspartate amino transferase and total bilirubin in petrol attendants were not elevated from the corresponding value in control group. There was no significant difference in liver function test in two different exposure groups, ie  $<10$  or  $>10$  years.

**Figure 5**

Table 2: Parameters of liver function test in petrol attendants and controls

Subject	N	Total Protein (g/dL)	Albumin (g/dL)	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	Total bilirubin (mg/dL)
Control (Confidential Interval)	33	7.31±3.23 (6.67-7.95)	5.25±0.21 (5.04-5.46)	165±34 (158-172)	63±13 (54-72)	40±9 (33-47)	0.77±0.4 (0.74-0.8)
Exposed group (Confidential Interval)	154	7.98±2.86 (6.75-9.21)	5.10±0.32 (4.8-5.4)	135±23 (122-148)	41±11 (37-45)	42±8 (38-44)	0.79±0.3 (0.75-0.77)
t, p value		0.68, 0.678	0.234, 0.872	342, 0.031	1.2, 0.045	1.6, 0.512	0.09, 0.876

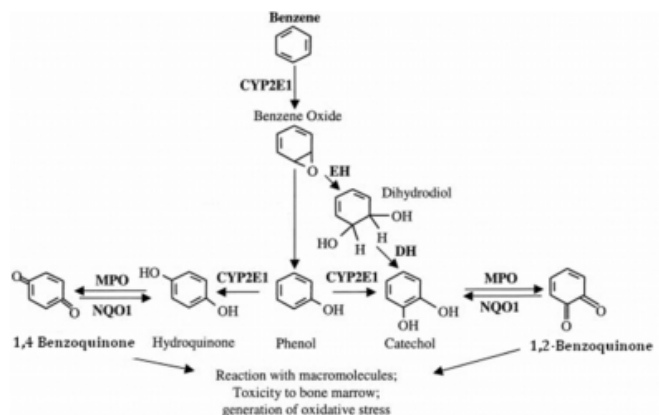
**DISCUSSION**

The aromatic hydrocarbon benzene is used in many different industrial activities and is present in fuels like petrol at a concentration of 0.5- 1.5%. The physiochemical properties of benzene include low evaporation temperature and vapor pressure allows its incorporation into the environment directly from the petrol industry or from automobiles. The chronic toxicity of benzene is the result of a series of biotransformation events that initiate with the generation of reactive intermediates which form covalent adducts with diverse critical macromolecules such as proteins and nucleic acids in liver, kidney, spleen and blood. The liver has the highest concentration of enzymes to metabolize carcinogens and toxicants. The most of the enzymatic reactions that convert lipophilic substances to hydrophilic conjugates in liver.

Majority of benzene metabolism occurs in the liver by cytochrome p4502E1. The major hepatic benzene metabolites are phenol, catechol, hydroquinone and trans trans muconic acid (fig: 4). The latter is presumed to be formed from the ring opening of benzene epoxide via benzene oxepin and has genotoxic properties and play a role in benzene toxicity. Thus, we evaluated the toxicity of benzene in lymphocytes and hepatocytes by both in vitro and in vivo methods.

**Figure 6**

Figure 4: Major metabolic pathway of benzene (legend: CYP, cytochrome P450; MPO, myeloperoxidase; NQO1, quinone oxidoreductase; EH, epoxide hydrolase; DH, dihydrodiol dehydrogenase).



Lymphocytes are uniquely sensitive to the toxic effects of benzene and its metabolites and are possibly one of the more sensitive indices to assess benzene associated toxicity. Depending on the dose and duration of exposure or time of exposure it covers a variety of effect that in general may be terms as toxic. Previous study indicated that exposure to benzene acutely affect human hematopoiesis system which leads to increased risk of leukemia at levels below 10ppm [19]. Kirkeleit et al (2008) also indicated that the benzene exposure to 10ppm in shoe manufacturing workers lead to deleterious effect on the lymphocytes [19]. To investigate this possibility, we proformed in vitro study to assess the viability of cultured spenic lymphocytes with different concentrations of benzene (1-10ppm). We observed a significant reduction in the viability of lymphocytes in a concentration depended manner. This observation indicates that the toxicity of benzene and its metabolites to lymphocytes was increasing as the concentration of the benzene in the culture medium increases. To further emphasize this, we evaluated the lymphocytes in workers exposed to benzene. When compared with the controls, significant reduction was observed in absolute count and percentage of lymphocytes in exposed group. Goldwater et al (1941) reported that lymphocytopenia was more frequently found among subjects exposed to benzene [20]. Lin et al (1998) reported that the absolute lymphocyte count and leukocyte count were similarly reduced in workers exposed only to benzene [21]. Studies by Wierda and Iron (1982) stated that the benzene and its metabolites (HQ and catechol) are immunotoxic, and results in potent B cell suppression as well as a block in B cell differentiation or maturation [22-24]. Studies conducted by Aoyama (1986) on

experimental animals reported that exposure to benzene develops a progressive B cell lymphocytopenia [25]. These observations were consistent with our results.

The liver as the key organ of metabolism and excretion, is constantly endowed with the task of detoxification. Hepatotoxicants including environmental pollutants and industrial solvents can induce various disorders of the organ. Metabolism in the liver protects tissues in higher organism from potentially harmful blood-borne environmental chemicals. Ironically, the metabolic products of detoxification reactions that protects other tissues from effects of the primary toxicant can be destructive to the liver when in excess or chronically present. In vitro mammalian hepatocyte culture still represents a compulsory step in the evaluation of toxic compounds because they carry out modifications leading to the production of various metabolites, which are the ultimate cause of toxicity. As the differentiated hepatocytes in cell culture function as like in the liver, they metabolize benzene to its corresponding metabolites, which are highly toxic to cell system. We evaluated the effect of benzene on the viability of cultured hepatocytes. Significant decrease in viability of cultured hepatocyte was observed with increase in concentration of benzene.

No studies have reported the effect of benzene on the human liver; however several animal studies were identified. The study conducted by Constan et al. (1996) evaluated apoptosis in the livers of Fischer-344 rats following exposure to drinking water containing seven contaminants, one of which was benzene [26]. Repeated exposure to these contaminants lead to the apoptosis which was directly correlated with changes in cell proliferation. Pawar and Mungikar (1975) reported an increase in liver weight after administering benzene to rats [27]. They also reported a decrease in protein in the post mitochondrial supernatant fractions, changes in hepatic drug metabolism and lipid peroxidation in the benzene exposed animals. Ugurnal et al. (1995) investigated the effect of acute and chronic benzene treatment on the lipid peroxidation and antioxidant system in mouse liver [28]. Malondialdehyde and diene conjugate levels were found to be increased in liver homogenates and microsomes of chronically exposed mice and were unchanged in the acutely exposed; glutathione levels remained unchanged in liver homogenates of all treatment groups. Whereas negative results were reported by Shell (1992), no adverse hepatic effects were observed in B6C3F1 mice exposed benzene in drinking water for 30 days [29].

The regular panels of LFTs are estimation of total protein (TP), albumin (Alb), (ALT), (ALP), total bilirubin (Tbil), (AST), gamma glutamyl transpeptidase and coagulase tests. Hepatic damage induced by any toxins can be observed by evaluating serum TP, AST, ALP and ALT levels. As these enzymes are cytoplasmic in nature, upon liver injury, these enzymes enter into the circulatory system due to altered permeability of membrane [30]. The present study reports that significant decrease in these two enzyme levels that may be due to inflammatory conditions in the benzene exposed groups. The liver produces most of the plasma proteins in the body and albumin is a protein made specifically by the liver. Albumin is decreased in chronic liver disease, nephritic syndrome, a state of poor nutrition and during protein catabolism. TP and Alb were slightly raised within the normal range in petrol attendants, thus revealing that they are not having any of the above diseases. ALP, an enzyme in the cells lining the biliary ducts of the liver, which will increases in the serum during obstructive liver disease. Significant low levels of ALP was observed in study group, which indicates absence of bile duct obstruction. During inflammatory conditions and acute liver damage, ALT rises dramatically. The statistically significant changes in the levels of ALT in study group than in controls indicate changes in the liver function which may be due to inflammation and it is supported by our in vitro cell culture studies, illustrating that as the concentration of benzene increases, inflammation as well as the toxicity increases. AST, another enzyme associated with liver parenchymal cells and is also present in red cells and cardiac muscle, which will increases in liver damage. Since AST is not specific for the assessment of liver function, slightly raised AST in study group might be due to gradual lyses of red blood cells (RBC). Bilirubin is measured to diagnose liver diseases. When RBC lyse, hemoglobin (Hb) is broken down within the macrophages to heme and globin, which further degraded to Fe<sup>2+</sup>, CO and bilirubin via the intermediate compound biliverdin. Since bilirubin is poorly soluble in water, it is carried to the liver bound to albumin. Slight increase in the levels of total bilirubin in study group when compared to controls might be an indication of hemolysis.

## **CONCLUSION**

In summary, this is the first study to have found a consistent relation between exposure to benzene and reduced hepatocyte functioning across a number of petrol attendants who were exposed to benzene. The results presented here also suggest significant toxicity to lymphocyte by in vitro method and lymphocytopenia in benzene-exposed



populations as well. In order to prevent these among petrol filling workers, we suggest that medical observation, including pre-employment and periodic medical checkups, should be performed which include liver function test. Thus medical screening and screening of ambient air concentration of benzene in air may protect workers from developing chronic hematological and liver disorders.

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