Biochemical Pharmacology, Vol. 33, No. 13, pp. 2009–2012, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

INVOLVEMENT OF IRON AND FREE RADICALS IN BENZENE TOXICITY

WASIUDDIN A. KHAN, ADITI GUPTA, UMA SHANKER and KUMUDRAY P. PANDYA*
Industrial Toxicology Research Centre, Lucknow, India

(*Received* 16 *August* 1983; *accepted* 3 *January* 1984)

Abstract—The ^{59}Fe distribution after a single i.v. injection of ^{59}Fe citrate in rats exposed to benzene was studied in circulating erythrocytes and organs up to period of 1 hr to 14 days. The iron content was significantly higher in bone marrow and liver compared to a control group of animals. A few cells with hemosiderin granules were observed in the benzene-administered group. Benzene increased lipid peroxidation in the liver and bone marrow and iron accelerated it further. Superoxide dismutase activities measured in terms of epinephrine auto-oxidation, an indirect measure of superoxide anion generation was enhanced in the benzene-treated groups. The data suggest the involvement of oxygen activation in benzene toxicity.

Prolonged exposure to benzene leads to progressive degeneration of the haemopoietic system. Toxic manifestation is characterized by depression of bone marrow function [1], which may be expressed as leucopenia and/or thrombocytopenia eventually leading to pancytopenia and aplastic anaemia [2]. Green *et al.* [3] evaluated the effects of benzene inhalation on the multipotential haematopoietic system and granulocyte/macrophage progenitor cells in mice. Marrow and splenetic cellularities were reduced. Red blood cell (RBC) counts were depressed and nucleated RBC in the marrow and spleen were also depressed. Granulocytopenia and lymphocytopenia were observed at 100 ppm. Infante *et al.* [4] obtained conclusive evidence from a cohort study of pilo film workers that there is a five-fold increased risk of leukaemia amongst chronically benzene-exposed workers. Based on a review of the entire set of studies, the accumulated evidence is conclusive that benzene exposure is related to the induction of leukaemia, various forms of cytopenia, aplastic anaemia and to the development of chromosomal aberrations.

Freedman and Rosman [5] observed the inhibition of haem synthesis in rabbit reticulocytes by benzene. Lee *et al.* [6] measured benzene-induced haem toxicity by measuring ^{59}Fe incorporation into circulating mice erythrocytes, which was reduced in 24 hr. Similar studies from our laboratory on ^{59}Fe incorporation into circulating erythrocytes of rats indicated that the rate of reappearance of ^{59}Fe in RBC was significantly decreased in the benzene-administered group of animals and radioactive iron content had accumulated in the target organs [7, 8]. The present study shows the relative distribution of ^{59}Fe in various organs at different time intervals. Attempts have been made to elucidate the role of iron and its correlation with enhanced lipid peroxidation and superoxide anion generation in bone marrow during benzene toxicity.

MATERIALS AND METHODS

Female albino rats (Swiss Wistar strain, bred in ITRC colony) weighing approximately 100 ± 10 g and maintained on a standard diet were used throughout the experiments. The animals were divided into seven groups, namely control (normal saline-administered) and benzene-administered (0.5 ml/kg body wt, i.p.) groups, with at least six animals being maintained in each group for different time intervals and placed individually in metabolic cages. Twenty-four hours after the last injection, all the animals were injected with a dose of ^{59}Fe (BARC) as ferric citrate (200 μg/0.2 ml per 100 g body wt) into a subclavian vein under light ether anaesthesia as described earlier [8].

At intervals ranging from 1 to 336 hr after the initial injection of ^{59}Fe, blood samples were collected from the tip of the tail in heparinized capillary tubes and were immediately transferred into weighed counting vials and reweighed. For the plasma, blood samples were centrifuged for 5 min. The capillary tubes were broken into a preweighed counting vial and reweighed. The plasma mass was converted to volume (using 1.025 as the specific gravity of the plasma).

After bleeding, animals from each group were killed by a blow on the head. Organs were immediately removed, cleaned and freed of extraneous materials, blotted on filter paper and weighed. All the samples were directly counted in an LKB-1280 Ultro Gamma II spectrometer using a ^{59}Fe window with an energy level of 60~70 keV at channel II. Care was taken tc ensure the same counting geometry for all samples.

For the estimation of iron and superoxide dismutase, the animals were divided into four groups comprising seven animals each. Animals in the first group were given a singe i.p. injection of benzene (0.5 ml/kg body wt) and were killed after 48 hr. The

* To whom correspondence should be addressed at: Industrial Toxicology Research Centre, M.G. Marg, Post Box No. 80, Lucknow 226001, India.

second group of animals were saline-administered controls. Animals in the third group were given benzene daily (0.5 ml/kg body wt) for 7 days, and in the fourth group, animals were given saline for the same period and were sacrificed by decapitation 24 hr after the last injection.

Assay of lipid peroxidation. Malonaldehyde levels as a measure of lipid peroxide formation were estimated by the procedure of Utley *et al.* [9]. Iron in the form of $FeCl_3$ was added to the incubation mixture to give the desired concentration. Iron as ferric citrate was also added to confirm the results.

Estimation of iron in the tissue. The iron content of the tissue was estimated by the method of Thompson and Blauchflower [10], and finally measured in a Perkin–Elmer Atomic absorption spectrophotometer (Model 5000).

Preparation and assay of bone marrow superoxide dismutase. Pooled bone marrow samples of approximately 300 mg were collected from the femurs of rats and were suspended in 3.0 ml of balanced salt solution of the same composition as that of Hanson *et al.* [11]. The purification procedure was slightly modified as follows. The suspension was homogenized in the same medium and centrifuged at 400 *g* for 10 min. The supernatant was again centrifuged at 9000 *g* for 25 min. The resulting pellets were suspended in 0.1 M phosphate buffer, pH 7.4. The post-mitichondrial supernatant and mitochondrial pellet suspensions were both subjected to the same treatment up to precipitation with KH_2PO_4 and directly used for dialysis.

The enzymes obtained were used for assay following the procedure of Misra and Fridovich [12] using twice the adrenaline concentration.

Staining for iron in bone marrow. Bone marrow slides from control and experimental animals were prepared and stained by Perl's Prussian blue method of hemosiderin using basic fuschin as a counter-stain [13].

RESULTS

The relative distribution of ^{59}Fe in the blood and plasma of normal and benzene-administered rats up to 14 days was studied following single intravenous injections of ^{59}Fe. There was little delay in reaching the maximum reappearance of ^{59}Fe in erythrocytes of benzene-treated groups (195 hr) compared to the control groups of animals (150 hr). The relative distributions of ^{59}Fe in the whole organs at various time intervals are given in Table 1. It appears that radioactive iron content is significantly high in the bone marrow and liver compared to the control group of animals. The maximum relative difference in iron content was noted between the control and benzene-treated group of animals at the end of 48 hr. The radioactive iron concentrations at the end of 48 hr in the bone marrow, liver and spleen were 3.9, 1.61 and 2.1 times more than that of the controls.

Table 2 gives the amount of iron present in the bone marrow and liver tissues as estimated by atomic absorption spectroscopy, which is in agreement with the preceding observation. Bone marrow tissues were stained for iron histochemically. A few normoblast and target cells were seen with hemosiderin

Table 1. Time-dependent distribution of ^{59}Fe after single treatment of benzene (0.5 ml/kg body wt) in rats (% of dose/g wet wt of tissue)

Time (hr)	Group	Organs				
		Bone marrow	Liver	Spleen	Brain	Kidney
12	Control	6.56 ± 0.57	6.18 ± 0.71	5.68 ± 0.46	0.11 ± 0.01	2.13 ± 0.22
	Experimental	5.50 ± 0.74	9.78 ± 1.52*	2.26 ± 0.36†	0.11 ± 0.02	4.22 ± 0.73‡
24	Control	6.50 ± 0.84	6.43 ± 1.50	4.13 ± 1.13	0.12 ± 0.01	2.67 ± 0.36
	Experimental	18.70 ± 1.17	7.31 ± 2.43	3.02 ± 0.38	0.10 ± 0.01	2.80 ± 0.31
48	Control	3.55 ± 0.75	2.25 ± 0.40	1.08 ± 0.07	0.059 ± 0.004	0.86 ± 0.21
	Experimental	13.90 ± 1.15†	3.62 ± 0.22	2.35 ± 0.19†	0.11 ± 0.01*	1.27 ± 0.07
72	Control	1.44 ± 0.10	3.93 ± 0.75	0.673 ± 0.05	0.054 ± 0.01	1.23 ± 0.13
	Experimental	2.42 ± 0.437	4.50 ± 0.37	0.78 ± 0.1	0.094 ± 0.017	1.48 ± 0.137
96	Control	0.46 ± 0.10	1.58 ± 0.13	0.55 ± 0.15	0.075 ± 0.02	0.55 ± 0.10
	Experimental	0.77 ± 0.03§	2.44 ± 0.50	0.995 ± 0.04	0.123 ± 0.03	1.17 ± 0.30
14 days	Control	0.50 ± 0.08	3.06 ± 0.57	0.80 ± 0.09	0.14 ± 0.02	1.15 ± 0.08
	Experimental	0.79 ± 0.11	2.15 ± 0.39	1.58 ± 0.40	0.15 ± 0.02	1.19 ± 0.15

* $P < 0.01$. ‡ $P < 0.02$.
† $P < 0.001$. § $P < 0.05$.

Table 2. Effect of i.p. benzene administration (0.5 ml/kg body wt) on the iron content of liver and bone marrow

Dose	Group	μg/g wet wt Bone marrow	Liver
Single injection	Control	152 ± 4	105 ± 6
	Experimental	192 ± 9*	120 ± 8
Seven injections	Control	172 ± 8	98 ± 6
	Experimental	260 ± 14*	157 ± 10*

* $P < 0.001$.
Data represent mean ± S.E. of seven animals.

granules in the benzene-administered bone marrow tissue. Diffused iron particles were also seen between the marrow cells.

Table 3 gives the *in vitro* effects of benzene and iron on bone marrow lipid peroxidation. It was observed that iron significantly enhanced it. Addition of catalase and superoxide dismutase reduced the observed increase in lipoperoxidation.

The superoxide dismutase activities of the bone marrow mitochondrial and post-mitochondrial fractions, measured in terms of the inhibition of epinephrine auto-oxidation, were enhanced in the benzene-treated group, indicating an increased generation of superoxide anions (Table 4).

DISCUSSION

Greenlee *et al.* [14] suggested that O_2^- generated by the auto-oxidation of benzene may be an important factor in benzene-induced cytotoxicity. It may be possible that the bone marrow is particularly sensitive to the lipid peroxide radical or superoxide anion *in situ*. The increase in the superoxide dismutase activities in bone marrow suggests that there is an increase in the formation of the superoxide anion (O_2^-). The increase in lipid peroxidation also suggests free radical formation in the target tissues. Ghoshal and Recknagel [15] obtained positive evidence of the activation of lipid peroxidation in rat liver by carbon tetrachloride.

The delay in the reappearance of ^{59}Fe in circulating erythrocytes in the benzene-administered group has been observed earlier [7, 8]. Snyder *et al.* [16] used the depression in ^{59}Fe uptake by RBC as an index of benzene-induced haematotoxicity.

The role of iron in the formation of reactive intermediate(s) also needs further studies. In the present study, a significant increase in radioactive iron content of bone marrow and liver was observed. This was also confirmed by atomic absorption spectroscopy. This increase may be due to the inhibition of δ-ALAD and increases in δ-ALAS activities after benzene administration [17]. The consequence of excessive iron overload on the basis of oxidation and free radical reactions may provide lesions of deregulated oxygen activation in benzene toxicity. The increase in lipid peroxidation in bone marrow and hepatic microsomes [18] by the addition of iron ions suggests similar phenomena in benzene intoxication also.

The ultrastructural and biochemical pattern in iron overload can be summarized as an increase in ferritin in the cytoplasm and its deposition in lysosomes, which may subsequently be transformed to hemosiderin [19]. Hence, iron can become decompartmentalized in such a situation. This may lead to the production of free radicals which result in tissue damage, as indicated by the peroxidation of membrane lipids. Iron is ideally suited to play the role of catalyst in the sequence of Haber–Weiss and Fenton-type reactions leading to the production of hydroxyl radicals [20]. The reaction can occur if sufficient concentrations of peroxide accumulate, which can be most damaging if it occurs in the vicinity of vital molecules such as nucleic acids [21]. Excessive accumulation of iron has been suggested to cause disaggregation of polyribosomes in maturing reticulocytes [22]. How-

Table 3. Acceleration of bone marrow lipid peroxidation by benzene and iron *in vitro*

Sample No.	Assay system	Levels of lipid peroxidation (nM MDA formed/30 min per mg protein)
1	Control	0.137 ± 0.010
2	Control + C_6H_6 (20 μl)	0.302 ± 0.046
3	+Fe + C_6H_6 (20 μl)	1.504 ± 0.061*
4	+Fe + C_6H_6 (20 μl) + catalase (3600 U)	0.361 ± 0.038†
5	+Fe + C_6H_6 (20 μl) + superoxide dismutase (5400 U)	1.01 ± 0.056
6	+Fe‡	1.14 ± 0.082*

Each value represents mean ± S.E. of seven individual determinations.
* Significantly higher as compared to control ($P < 0.001$).
† Significantly inhibited as compared to sample No. 3 ($P < 0.01$), as evaluated by Student's *t*-test.
‡ 2.5 mM $FeCl_3$ or Fe citrate.

Table 4. Inhibition of auto-oxidation of adrenaline by bone marrow superoxide dismutase preparation (activity expressed as μM adrenochrome formed/min per 25 μg protein)

Group	Mitochondrial enzyme	Post-mitochondrial enzyme
Single injection		
Control	2.88 ± 0.03	3.05 ± 0.04
Experimental	0.596 ± 0.065*	1.96 ± 0.05*
Seven injections		
Control	2.88 ± 0.03	3.05 ± 0.04
Experimental	1.365 ± 0.133*	2.23 ± 0.039*

The values shown were calculated using $E_{max} = 4.01 \times 10^3 M^{-1}cm^{-1}$ and background. Epinephrine oxidation was 3.75 μmole/min. Superoxide dismutase in semipurified preparation contained 25 μg protein. All values represent mean ± S.E. of seven animals.

* $P < 0.001$, as evaluated by Student's *t*-test.

ever, *in vivo* evidence is not yet available. The decrease in lipoperoxidation by catalase and superoxide dismutase suggests the involvement of hydroxyl radicals/superoxide anions.

Present data suggest that the elevated generation of superoxide anion/lipid peroxide radicals during benzene toxicity may be involved in the formation of hydroxyl radical mediated cytotoxic products.

Acknowledgements—We are grateful to the Director of the Centre for his interest. W.A.K. and A.G. are grateful to the Council of Scientific and Industrial Research for the award of a research fellowship.

REFERENCES

1. M. Ikeda and H. Ohtsuji, *Toxic. appl. Pharmac.* **20**, 30 (1971).
2. P. N. Cheremisinoff and A. C. Morresi, in *Benzene: Basic and Hazardous Properties* (Eds. R. A. Yound and P. N. Cheremisinoff), p. 95. Marcel Dekker, New York (1979).
3. J. D. Green, C. A. Snyder, J. Lobue, B. D. Goldstein and R. E. Albert, *Toxic. appl. Pharmac.* **59**, 204 (1981).
4. P. F. Infante, R. A. Rinsky, J. K. Wagoner and R. J. Young, *Lancet* **ii**, 76 (1977).
5. M. L. Freedman and J. Rosman, *J. clin. Invest.* **57**, 594 (1976).
6. E. W. Lee, J. Kocsis and R. Snyder, *Res. Commun. chem. Path. Pharmac.* **5**, 547 (1973).
7. S. Khan and K. P. Pandya, *Proc. Soc. Biol. Chem. (India)* **A-267**, 112 (1978).
8. R. C. Srivastava, S. Khan, U. Shankar and K. P. Pandya, *Archs Toxic.* **42**, 43 (1979).
9. H. G. Utley, F. Bernheim and P. Hochstein, *Archs Biochem. Biophys.* **118**, 29 (1967).
10. R. H. Thompson and W. J. Blauchflower, *Lab. Pract.* **20**, 849 (1971).
11. S. K. Hanson, H. W. Anders and M. R. Montgomery, *Biochem. biophys. Res. Commun.* **85**, 783 (1978).
12. H. P. Misra and I. Fridovich, *J. biol. Chem.* **247**, 3170 (1972).
13. E. Kaplan, W. W. Zuelzer and C. Mouriquand, *Blood* **9**, 203 (1954).
14. W. F. Greenlee, J. D. Sun and J. S. Bus, *Toxic. appl. Pharmac.* **59**, 187 (1981).
15. A. K. Ghoshal and R. O. Recknagel, *Life Sci.* **4**, 1521 (1965).
16. R. Snyder, S. L. Longacre, C. M. Witmer, J. J. Kocsis, L. S. Andrews and E. W. Lee, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), Vol. 3, p. 123. Elsevier/North Holland, Amsterdam (1981).
17. K. Chandra, A. Gupta and K. P. Pandya, *Proc. Soc. Biol. Chem. (India)* **S-1**, 19.02 (1981).
18. K. P. Pandya, W. A. Khan, A. Gupta and Umashankar, *Toxic. Lett.* **18**, 97 (1983).
19. R. R. Crichton, in *Oxygen-Free Radicals and Tissue Damage* (Ed. D. W. Fitzsimons), p. 57. Ciba Foundation Symposium 65. Excerpta Medica, Amsterdam (1979).
20. R. L. Wilson, in *Oxygen-Free Radicals and Tissue Damage* (Ed. D. W. Fitzsimons), p. 19. Ciba Foundation Symposium 65. Excerpta Medica, Amsterdam (1979).
21. J. W. Lawn and S. Sims, *Biochem. biophys. Res. Commun.* **77**, 1150 (1977).
22. H. S. Waxman and M. Ravinovitz, *Biochim. biophys. Acta* **129**, 369 (1966).