

of *N*-nitrosopiperazine derivatives
 piperidine and *N*-dinitrosopiperazine
)-651.
 focus of yeast, Diss. Abstr. Int., 33

of *Saccharomyces cerevisiae*: Fin
) 35-51.
 osopiperazines for *Salmonella typhi*
 -1599.

Mutation Research, 77 (1980) 149-155
 © Elsevier/North-Holland Biomedical Press

THE EFFECT OF BENZENE IN THE MICRONUCLEUS TEST

MARK HITE, MARIE PECHARO, INEZ SMITH and SUSAN THORNTON

*Department of Safety Assessment, Merck Institute for Therapeutic Research, Merck Sharp
 and Dohme Research Laboratories, West Point, PA 19486 (U.S.A.)*

(Received 25 April 1979)

(Revision received 17 August 1979)

(Accepted 3 October 1979)

Summary

The potential cytogenetic effect of benzene was studied by means of the micronucleus test in developing erythrocytes of male and female mice at dosages ranging from 0.0625 to 2.0 ml/kg/day. Groups of mice were given two daily oral doses and sacrificed 6, 18, 24 or 48 h, or 5, 9 or 16 days after the last dose. The doses were given 24 h apart. Bone marrow from the mice was processed for visualization of micronuclei in polychromatic erythrocytes.

The results showed a significant ($P < 0.05$) increases in the numbers of polychromatic erythrocytes with micronuclei from mice sacrificed 6 h after being given dosages of 0.25 ml/kg/day or more of benzene. Similarly, significant ($P < 0.05$) increases in polychromatic erythrocytes with micronuclei were seen in mice given 0.125 ml/kg/day or more of benzene and sacrificed 18 or 24 h after the second dose. However, in a second experiment negative results were obtained at 24 h from mice at 0.0625, 0.125 and 0.25 ml/kg. One group of mice (0.25 ml/kg/day and killed at 48 h) showed significant increases in the numbers of polychromatic erythrocytes with micronuclei. Mice killed 5 days after treatment with 2 doses of 0.125 ml/kg or more of benzene showed significant numbers of micronuclei in polychromatic erythrocytes. The groups of mice at 0.5, 1.0 or 2.0 ml/kg/day and killed 9 or 16 days after the second dose of benzene showed values that were comparable to the corresponding negative controls. In all cases there were sufficient numbers of cells to analyze. Although benzene is a known hematopoietic poison and bone-marrow depressant, there were sufficient numbers of cells to evaluate in the micronucleus test. Thus, these experiments uphold the usefulness of the micronucleus test as a screening procedure in cytogenetics.

During the development of the micronucleus test for inclusion in a short-term carcinogenic/mutagenic testing program, several reference compounds

TABLE 1
— AT EACH TIME POINT

Treatment group	Time sacrificed after second dose ^c						
	6 h ^b	18 h	24 h ^b	48 h	Day 5	Day 9	Day 15
Negative control, peanut oil 10 ml/kg/day, p.o.	13	10	10	10	—	—	—
Positive control, MMS 90 mg/kg/day, i.p.	4	—	—	—	—	4	4
0.0625 ml/kg/day, p.o., benzene ^a	—	10	10	10	—	—	—
0.125 ml/kg/day, p.o., benzene ^a	—	10	10	10	10	—	—
0.25 ml/kg/day, p.o., benzene	—	10	10	10	10	—	—
0.5 ml/kg/day, p.o., benzene	8	—	—	—	10	8	—
1.0 ml/kg/day, p.o., benzene	8	—	—	—	—	8	—
2.0 ml/kg/day, p.o., benzene	8	—	—	—	—	8	8

—, Not done.

^a Groups were equally divided by sex.

^b Two experiments were done at 6 and 24 h.

^c Each dose was given 24 h apart.

were employed in our laboratory and hematopoietic poison [2,7,11] years, evidence has accumulated from industrial workers where information from clinical and epidemiologic studies for Occupational Safety and Health as a genotoxic agent [13]. The reason for the malignant disease of the blood. It recommends that for regulatory purposes in humans. The Consumer Product Commission in the Federal Register [5,14]. OSHA's conclusions that employment is a hazard, specifically the hazard of benzene.

The micronucleus test is based on the observation that in the cytoplasm of young cells previously mentioned benzene treatment seemed worthwhile to investigate its effect in this test.

Materials and methods

The potential cytogenetic effect of the micronucleus test in developing experiments as outlined in Table 1. The procedure of Schmid [14,15] was used.

The mice used in the various experiments were of the C57BL/6J strain. They were about 5 weeks old. The animals were maintained in standard conditions in a temperature-controlled room (about 22°C) with 12 h of light and 12 h of dark, with water at all times.

The benzene used in these experiments was of analytical grade (Fisher Scientific). It was diluted (1:5) in an equal volume of mineral oil (Raynard, Inc.). The solution was stored in glass syringes and stainless steel syringes. The mice received an equivolumetric dose of benzene.

In 3 experiments, a positive control was used. This compound was dissolved in mineral oil at a dose of 0.0625 ml/kg (2.6 mg/ml) at a dose of 0.0625 ml/kg (2.6 mg/ml) benzene-treated animals. The control was starting at approx. one-third of the dose of 0.0625 ml/kg or 5.35 ml/kg, for mice [3].

The mice were sacrificed by cervical dislocation 7 days after the second dose. Blood was collected from each mouse by centrifugation in a microfuge tube, using fetal calf serum (FCS) 5 min at 1000 rpm. The supernatant was then used for the micronucleus test.

were employed in our laboratory. Benzene, a known bone-marrow depressant and hematopoietic poison [2,7,17], was one of these compounds. In recent years, evidence has accumulated implicating benzene in the etiology of leukemias in industrial workers where benzene was used heavily [4,9,12,18]. This information from clinical and epidemiologic studies led the National Institute for Occupational Safety and Health (NIOSH) to identify benzene as a leukemogenic agent [13]. The reason for this is that the chemical causes progressive, malignant disease of the blood-forming organs, and as such, NIOSH recommends that for regulatory purposes benzene be considered carcinogenic in humans. The Consumer Product Safety Commission (CPSC) and the Occupational Safety and Health Administration (OSHA) published benzene standards in the Federal Register [5,14]. The basis for the standards were CPSC's and OSHA's conclusions that employee exposure to benzene presents a cancer hazard, specifically the hazard of developing leukemia.

The micronucleus test is based on the detection of small particles of chromatin in the cytoplasm of young (24–30 h old) erythrocytes [8,15,16]. As previously mentioned benzene is a known bone-marrow depressant, thus it seemed worthwhile to investigate a chemical with this type of biological activity in this test.

Materials and methods

The potential cytogenetic effect of benzene was studied by means of the micronucleus test in developing erythrocytes of male and female mice in several experiments as outlined in Table 1. Each test was done following the reported procedure of Schmid [14,15] with minor modifications.

The mice used in the various experiments were of the Charles River (CD-1) strain. They were about 5 weeks old and weighed between 10 and 20 g. The animals were maintained in standard clear plastic mouse boxes in an air-conditioned room (about 22°C) with free access to food (Purina Lab Chow) and water at all times.

The benzene used in these experiments was of reagent grade (Fisher Scientific). It was diluted (1:5) in an edible grade of peanut oil (Magnus, Mahee and Raynard, Inc.). The solution was given to the mice by oral intubation using glass syringes and stainless steel gavage tubes. The negative control groups of mice received an equivolumetric amount of peanut oil (10 ml/kg).

In 3 experiments, a positive control agent, methyl methanesulfonate (MMS) was used. This compound was injected intraperitoneally in mice as an aqueous solution (2.6 mg/ml) at a dose of 90 mg/kg under a regimen similar to the benzene-treated animals. The dosages of benzene were chosen by arbitrarily starting at approx. one-third of the reported oral LD₅₀ value, which is 4700 mg/kg or 5.35 ml/kg, for mice [3]. The doses were given 24 h apart.

The mice were sacrificed by cervical dislocation 6, 18, 24 or 48 h, or 9 or 16 days after the second dose. Bone marrow from both femora was harvested and prepared for microscopic analysis after the method of Schmid [15]. The marrow from each mouse was evacuated from the marrow canal into a siliconized centrifuge tube, using fetal-calf serum, and the tubes were centrifuged for 5 min at 1000 rpm. The supernatant was removed with Pasteur pipettes, and

—, Not done.

a Groups were equally divided by sex.

b Two experiments were done at 6 and 24 h.

c Each dose was given 24 h apart.

TABLE 2

SUMMARY OF RESULTS FROM MICRONUCLEIUS TESTS

Treatment group	Polychromatic erythrocytes ^a with micronuclei	Percentage of (range) in percent	Day 5			
Time sacrificed after second dose ^b	4 h	48 h				
6 h	18 h					
Negative control, peanut oil 10 ml/g/day, p.o.	0.4 (0.07-0.72)	0.60 (0.13-1.13)	0.54 (0.19-1.02)	0.53 (0.25-1.13)	0.46 (0.12-1.07)	0.47 (0.15-0.8)
Positive control, fms 90 mg/kg/day, i.p.	47.8 ^d (24.1-49.0)	-	-	-	-	-
0.0625 ml/kg/day, o., benzene	-	0.62 (0.15-1.32)	0.76 (0.45-1.38)	0.60 (0.34-0.83)	0.34 (0.00-0.68)	-
0.125 ml/kg/day, o., benzene	-	0.78 (0.18-1.73)	1.8 ^u (0.5-1.70)	0.82 (0.23-1.4)	1.36 ^d (0.75-2.5)	1.36 ^d (0.76-2.13)
0.25 ml/kg/day, o., benzene	-	0.32 ^d (0.06-1.80)	1.33 ^d (0.47-2.71)	0.78 (0.17-1.55)	2.13 ^d (0.63-5.5)	2.13 ^d (0.66-5.1)
0.5 ml/kg/day, o., benzene	3.2 (0.5-7.36)	-	-	-	1.51 ^d (0.25-2.8)	1.51 ^d (0.26-2.6)
0.0 ml/kg/day, o., benzene	2.46 ^d (0.29-6.31)	-	-	-	-	-
0.0 ml/kg/day, o., benzene	5.24 ^d (1.28-10.68)	-	-	-	-	-

^a Not done.^b Normochromatic erythrocytes with micronuclei were counted, but the few as isolated in ship of treated mice to group point. Each dose was given 24 h apart.^c Two experiments were done at 6 and 2 h.^d Significantly greater than corresponding negative group; $P < 0.05$.

10.07-0.88 10.15-0.60

using a rankit transformation. Difference procedure for pairwise comparison of a treated group [7,17].

Results

The results of the various experiments represent averages and range of erythrocytes with micronuclei of each cell type from both male and female mice. Responses were similar in males and females seen in normochromatic erythrocytes.

All mice appeared in normal health. No sign of toxicity. Furthermore, sufficient numbers of cells to count were available.

The data show a significant increase in normochromatic erythrocytes with micronuclei at dosages of 0.25 ml/kg/day or higher. Increases in polychromatic erythrocytes were given 0.125 or 0.25 ml/kg/day dose.

Two separate experiments were done at doses of benzene at 0.0625, 0.125, 0.25, and 0.5 ml/kg/day. Increases in the number of micronuclei compared to the corresponding control doses 0.125, 0.25 and 0.5 ml/kg/day were significant.

With the exception of the 0.0625 ml/kg/day dose, significant ($P < 0.05$) increases in micronuclei were seen in normochromatic erythrocytes (0.0625 and 0.125 ml/kg/day).

Mice killed 5 days after treatment with 0.125 or 0.5 ml/kg/day showed significant increases in normochromatic erythrocytes with micronuclei. The groups of mice at 0.125

0.5 ml/kg/day, p.o., benzene	3.20 d (0.56—7.36)	—	—	1.51 d (0.26—2.68)	—	0.23 (0.00—0.43)	—
1.0 ml/kg/day, p.o., benzene	2.46 d (0.29—6.31)	—	—	—	—	0.50 (0.26—0.76)	—
2.0 ml/kg/day, p.o., benzene	5.24 d (1.28—10.68)	—	—	—	—	0.40 (0.07—0.88)	0.28 (0.15—0.60)

—, Not done.

a Normochromatic erythrocytes with micronuclei were noted, but there was no relationship to treatment at any point.

b Each dose was given 24 h apart.

c Two experiments were done at 6 and 24 h.

d Significantly greater than corresponding negative control group at $P \leq 0.05$.

the cells then were resuspended by mixing gently with siliconized pipettes in the small amount of serum remaining in the capillary part of the test tube. A drop of the cell suspension was placed on a clean microscope slide and spread in the conventional manner. 4 slides were prepared for each mouse. The smears were air-dried on a slide dryer at 37°C and dipped briefly into absolute methanol for fixation. The slides were stained with May-Gruenwald Giemsa stain. The microscope slides were coded so that they could be read in a blind and random fashion. Approx. 3000 polychromatic and normochromatic erythrocytes from each mouse were examined for the presence of micronuclei. Microscopic evaluation was done with brightfield oil immersion optics at 1250X.

The statistical analysis consisted of (a) the analysis of variance procedure using a rankit transformation of the data and (b) Fischer's Least Significant Difference procedure for pairwise comparison between the negative control and a treated group [7,171].

Results

The results of the various experiments are summarized in Table 2. The values represent averages and ranges of the frequency (per cent) of polychromatic erythrocytes with micronuclei compared to the total scored population of this cell type from both male and female mice in the various treatment groups. The responses were similar in male and female mice. The number of micronuclei in nonchromatic erythrocytes did not show any relationship to treatment.

All mice appeared in normal physical conditions and none showed any overt sign of toxicity. Furthermore, when the smears were examined there were sufficient numbers of cells to analyze from mice in all groups.

The data show a significant ($P < 0.05$) increase in the numbers of polychromatic erythrocytes with micronuclei from mice sacrificed 6 h after being given dosages of 0.25 ml/kg/day or more of benzene. Similarly, significant ($P < 0.05$) increases in polychromatic erythrocytes with micronuclei were seen in mice given 0.125 or 0.25 ml/kg/day of benzene and sacrificed 18 h after the second dose.

Two separate experiments were done at 24 h. In the first, mice given 2 daily doses of benzene at 0.0625, 0.125 and 0.25 ml/kg showed no significant ($P < 0.05$) increases in the number of polychromatic erythrocytes with micronuclei compared to the corresponding negative control. In the second test, 2 daily doses 0.125, 0.25 and 0.5 ml/kg produced significant ($P < 0.05$) numbers of micronuclei in the same type of cell.

With the exception of the mice at 0.25 ml/kg/day and killed at 48 h, no significant ($P < 0.05$) increases in the numbers of polychromatic erythrocytes with micronuclei were seen in the two other groups of benzene-treated mice (0.0625 and 0.125 ml/kg/day) sacrificed at this time period.

Mice killed 5 days after treatment with 2 oral doses of benzene (0.125, 0.25 or 0.5 ml/kg) showed significant ($P < 0.05$) increases in the numbers of polychromatic erythrocytes with micronuclei.

The groups of mice at 0.5, 1.0 or 2.0 ml/kg/day and killed 9 or 16 days after

the second dose benzene showed values that were comparable to the corresponding negative controls.

Discussion

Several compounds of varying chemical classes have been reported to increase the number of polychromatic erythrocytes with micronuclei in test animals [16,19]. Many of these agents are considered to be carcinogenic and have given positive results in studies with mammalian and submammalian systems [19]. This should be contrasted with a series of 16 carcinogens in which 11 gave no response in the micronucleus test in the rats [18].

As reported by Lyon [10] rats with 0.05 and 0.25 mg/kg of benzene on two successive days showed significantly increased numbers of micronuclei than corresponding controls. A dosage of 0.025 mg/kg for two days did not cause any noticeable change. Furthermore, benzene has been shown to cause chromosome aberrations in rats and rabbits [6] and in humans, both in vivo and in vitro [6,20].

The effects of benzene in the experiments reported herein were dose-related within the expected variability of the test. The results of this study, therefore support information about the biological activity of benzene since it is reported to be leukemogenic and clastogenic.

Although benzene is a known hematopoietic poison, there were sufficient numbers of cells in the bone marrow of treated mice to evaluate in the micronucleus test. These experiments uphold the usefulness of the micronucleus test as a screening procedure in cytogenetics. Of course, if the animals were dosed with benzene for several days, then the cell population in the bone marrow conceivably could be reduced to an unsatisfactory level for scoring in the micronucleus test.

Acknowledgement

The authors wish to acknowledge the statistical assistance of Dr. N.R. Bohidar and the technical encouragement of Dr. H.M. Peck, both of the Merck Sharp and Dohme Research Laboratories, West Point, PA. In addition, the authors express their appreciation to Mrs. Thelma Demetrius for secretarial assistance.

References

- 1 Bruckner, J.V. and R.G. Peterson, Toxicology of aliphatic and aromatic hydrocarbons, chapter 9: in National Institute on Drug Abuse Research Monograph Series 15, C.W. Sharp and M.L. Brehm (Eds.), Review of Inhalants: Euphoria and Dysfunction. 1977, pp. 124-163.
- 2 Christensen, H.E., and T.T. Luginbyhl, Registry of toxic effects of chemical substances, National Institute for Occupational Safety and Health, U.S. Dept. of Health, Education, and Welfare, Rockville, MD. 1975, p. 163.
- 3 Cohen, H.S., M.F. Freedman and B.D. Goldstein. The problem of benzene in our environment: Clinical and molecular considerations, Am. J. Med. Sci., 275 (1978) 125-136.
- 4 Consumer Product Safety Commission, Benzene, Federal Register 43 (1978). part IV, 21838-21854.
- 5 Dean, B.J., Genetic toxicology of benzene, toluene, xylenes and phenols, Mutation Res., 47 (1978) 75-97.
- 6 Goldstein, B.D., Hematotoxicity in humans, J. Toxicol. Environ. Health, Suppl. 2 (1977) 69-105.

- 7 Harter, H.L., Expected values
- 8 Heddle, J.A., and A.V. Carrai
γ-irradiation: Evidence that
44 (1977) 63-69.
- 9 Infante, P.F., J.K. Wagoner,
(1977) 76-78.
- 10 Lyon, J.P., Mutagenicity stud
- 11 Maltoni, C., Research "bombs
- 12 National Institute for Occup
Revised recommendation for
- 13 Occupational Health and Sa
benzene, Federal Register 42
- 14 Schmid, W., Chemical mutat
(1973) 77-85.
- 15 Schmid, W., The micronucle
Chemical Mutagens - Princip
pp. 31-53.
- 16 Snedecor, G.W., and W.G. Co
Iowa, 1967, p. 272.
- 17 Snyder, R., E.W. Lee, J.J.
actions of benzene, Life Sci.,
- 18 Trzos, R.J., G.L. Petzold, M
the rat using the micronucleu
- 19 Vigliani, E.C., and A. Forni, J
- 20 Wild, D., Cytogenetic effects
micronucleus test, Mutation
- 21 Wolman, S.R., Cytologic an
(1977) 63-68.

comparable to the control.

have been reported to show micronuclei in testis to be carcinogenic and in and submammary of 16 carcinogens in rats [18].

100 mg/kg of benzene on two days of micronuclei than two days did not cause micronuclei to cause chromosomal changes, both in vivo and in vitro.

wherein were dose-related of this study, therefore, benzene since it is reported to be a carcinogen.

therefore, there were sufficient data to evaluate in the micronucleus test of the animals were dosed in the bone marrow level for scoring in the micronucleus test.

with the assistance of Dr. N.R. Leach, both of the Merck and Co., Inc., Kenilworth, PA. In addition, the author wishes to thank the secretarial assistance of Dr. N.R. Leach.

polycyclic aromatic hydrocarbons. chapter 9: in: *Polycyclic Aromatic Hydrocarbons*, Sharp and M.L. Brehm (Eds.),

chemical substances, National Institute for Occupational Safety and Health, U.S. Dept. of Health, Education, and Welfare, Rockville, MD, 1977.

benzene in our environment: *Clinical Toxicology*, 6, 1978, part IV, 21838-21854.

mutagenesis, *Mutation Res.*, 47 (1978) 1-10.

Suppl. 2 (1977) 69-105.

- 7 Harter, H.L., Expected values of normal order statistics, *Biometrika*, **48** (1960) 151-165.
- 8 Heddle, J.A., and A.V. Carrano, The DNA content of micronuclei induced in mouse bone marrow by gamma-irradiation: Evidence that micronuclei arise from acentric chromosomal fragments, *Mutation Res.*, **44** (1977) 63-69.
- 9 Infante, P.F., J.K. Wagoner, R.A. Rinsky and R.J. Young, Leukemia in benzene workers, *Lancet*, **2** (1977) 76-78.
- 10 Lyon, J.P., Mutagenicity studies with benzene, Ph. D. thesis, University of California, 1975.
- 11 Maltoni, C., Research "bombshell" hits benzene, as reported in *Chem. Week*, **121** (1977) 33.
- 12 National Institute for Occupational Safety and Health, U.S. Dept. of Health, Education and Welfare, Revised recommendation for an occupational exposure standard for benzene, 1977.
- 13 Occupational Health and Safety Administration, U.S. Dept. of Labor, Occupational exposure to benzene, *Federal Register* **42** (1977) 27452-27461.
- 14 Schmid, W., Chemical mutagen testing on in vivo somatic mammalian cells, *Agents and Actions*, **3** (1973) 77-85.
- 15 Schmid, W., The micronucleus test for cytogenetic analysis, chapter 36 in: A. Hollaender (Ed.), *Chemical Mutagens - Principles and Methods for Their Detection*, Vol. 4, Plenum, New York, 1976, pp. 31-53.
- 16 Snedecor, G.W., and W.G. Cochran, *Statistical Methods*, 6th edn., Iowa State University Press, Ames, Iowa, 1967, p. 272.
- 17 Snyder, R., E.W. Lee, J.J. Kocsis and C.M. Witmer, Bone marrow depressant and leukemogenic actions of benzene, *Life Sci.*, **21** (1977) 1709-1722.
- 18 Trzos, R.J., G.L. Petzold, M.N. Brunden and J.A. Swenberg, The evaluation of sixteen carcinogens in the rat using the micronucleus test, *Mutation Res.*, **58** (1978) 79-86.
- 19 Vigliani, E.C., and A. Forni, Benzene and leukemia, *Environ. Res.*, **11** (1976) 122-127.
- 20 Wild, D., Cytogenetic effects in the mouse of 17 chemical mutagens and carcinogens evaluated by the micronucleus test, *Mutation Res.*, **56** (1978) 319-327.
- 21 Wolman, S.R., Cytologic and cytogenetic effects of benzene, *J. Toxicol. Environ. Health, Suppl.* **2** (1977) 63-68.