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Studies on three structurally related phenylenediamines with the mouse micronucleus assay system

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Summary

Three structurally related compounds, 4-chloro-*o*-phenylenediamine (COP), 4-nitro-*o*-phenylenediamine (NOP) and *p*-phenylenediamine dihydrochloride (PPD), are used in fur dyes, inks and hair coloring formulations. COP has been reported to be carcinogenic in both rats and mice. NOP and PPD are non-carcinogens, but have consistently tested positive in short-term in vitro genotoxicity assays. Studies were undertaken to evaluate their genotoxicity with the in vivo mouse bone-marrow micronucleus assay. Five CD-1 male mice per dose were injected i.p. with the compounds and sacrificed at intervals of 24, 48 and 72 h. 2000 cells were scored per animal to determine the frequency of micronucleated-polychromatic erythrocytes (MPCE). COP induced significant dose-related increases in MPCE over the 3 doses tested at each of the sampling intervals. The peak response occurred at 24 h. No response was observed in animals treated with PPD or NOP.

The phenylenediamines were defined by the Interagency Testing Committee (ITC) of the Toxic Control Substances Act as all nitrogen unsubstituted phenylenediamines with 0–2 substituents on the ring (Anonymous, 1980). The ITC recommended that these compounds including 4-chloro-*o*-phenylenediamine (COP), 4-nitro-*o*-phenylenediamine (NOP) and *p*-phenylenediamine dihydrochloride (PPD) should be evaluated through testing for carcinogenicity, mutagenicity, teratogenicity and other health effects because of their human exposure. COP is used as an oxidative base in dyes and as dye intermediate. Also, it is used to synthesize drugs as a chemical reagent. NOP and PPD are used as dyes in semipermanent and permanent hair dye formulations. They are also used as intermediates in the production of dyes for furs and inks (Anonymous, 1980).

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COP is carcinogenic in both mice and rats (Anonymous, 1980) and has been shown to cause tumors in the bladder, lung, mammary glands and thyroid gland (Hernandez, 1982). It is known to be mutagenic in bacteria (Anonymous, 1980; Dunkel and Simmon, 1980; Dunkel et al., 1985; Haworth et al., 1983; Hernandez, 1982) and induce DNA-strand breaks in cultured human fibroblasts (Nordenskjold et al., 1984). McFee et al. (1986) have shown that COP causes multiple aberrations in a few (2–8%) mouse bone-marrow cells. Yet, there was no increase in aberration rate among the remaining cells and no increase in sister-chromatid exchanges.

Both NOP and PPD have been shown to be non-carcinogenic by the National Cancer Institute (Anonymous, 1980) and the National Toxicology Program (Shelby and Stasiewicz, 1984). However, studies have shown that both compounds are mutagenic in the Ames Salmonella assay (Dunkel et al., 1985; Gentile et al., 1986; Haworth et al., 1983; Natarajan and Obe, 1986; Prival et al., 1984) and in the L5178YTK$^{+/-}$ mouse lymphoma assay (Oberly et al., 1984; Shelby and Stasiewicz, 1984). Moreover, NOP was found to be genotoxic in Drosophila sex-linked recessive lethal and yeast mitotic recombination assays (Natarajan and Obe, 1986). It has also been shown that both NOP and PPD induced chromosome aberrations and/or SCEs in vitro in mammalian cell cultures (Perry and Searle, 1977; Shelby and Stasiewicz, 1984). Negative results with NOP and PPD have been reported. Searle et al. (1975) found that in human lymphocytes, NOP did not induce chromosomal aberrations. In the hepatocyte primary culture/DNA-repair assay, both NOP and PPD gave negative results (Williams et al., 1982). Also, Natarajan and Obe (1986) reported that NOP did not increase micronucleus formation.

This study was aimed at determining if the micronucleus assay can distinguish between the carcinogen (COP) and the two noncarcinogens (NOP and PPD).

Materials and methods

Animals. Male CD-1 mice, 8–10 weeks old, weighing 25–30 g, were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). A commercial diet (Purina Certified Laboratory Rodent Chow) and water were provided ad libitum throughout the period of animal holding and experimentation.

Chemicals. 4-chloro-*o*-phenylenediamine, 4-nitro-*o*-phenylenediamine and *p*-phenylenediamine dihydrochloride were provided through the National Toxicology Program from Radian Corporation (Austin, TX). Triethylenemelamine (TEM) was purchased from Polysciences Laboratories (Warrington, PA). The solvent for COP and NOP was dimethyl sulfoxide (DMSO) while PPD was dissolved in sterile distilled water. TEM, at a concentration of 1.5 mg/kg, dissolved in sterile distilled water, was used as a positive control in all experiments. In each experiment solvent controls were routinely run.

The three compounds were tested at highest dose levels which allowed animals to survive the length of experiments. COP killed 4 of 5 mice at 500 mg/kg so 400 mg/kg was chosen. NOP killed 2 of 5 at 750 mg/kg so 500 mg/kg was used. PPD at 200 mg/kg killed 3 of 5 animals and 100 mg/kg was used as the highest dose. The medium and low doses were 1/2 and 1/4 that of the high dose. Animals were treated by intraperitoneal (i.p.) injection of test or control compounds with a volume of 0.2 ml per animal.

Sampling time. Three sampling times, 24, 48 and 72 h after injection, were used for test compounds. Both vehicle and positive controls were sampled only at 24 h post-treatment.

Micronucleus assay. At desired sampling intervals, mice were sacrificed by cervical dislocation. Femora were removed from each animal and ends of bones were cut off using bone cutters. Bone marrow was flushed from bones with 3 ml fetal bovine serum (FBS), into 15-ml centrifuge tubes. Cells were gently centrifuged at 1000 rpm for 5 min. The supernatant, except for a few drops, was removed. The cell pellet was resuspended and aspirated to obtain a homogeneous cell suspension. One drop of suspension was placed onto each clean, dry microscope slide and smears were made using beveled slides. Slides were allowed to air dry overnight and then fixed in absolute

methanol for 15 min. Bone marrow cells were stained with May–Gruenwald Stain for 17 min and then with 10% Azure B Giemsa for 5–6 min. After staining, slides were agitated for 10–20 sec in Gurr's buffer, rinsed in deionized water and allowed to air dry. When dry, slides were coded and ready to be scored.

Scoring. Criteria for scoring were those of Schmid (1975). 2000 polychromatic erythrocytes (PCEs) per animal were scored for incidence of micronuclei. The ratio of PCEs to normochromatic erythrocytes (NCEs) was also determined in the first 1000 PCEs scored per animal (Hart and Engberg-Pederson, 1983). A total of 5 animals per dose level was scored to give a total of 10000 PCEs scored per dose point.

Statistical analysis. When using 2000 cells per animal, previous data indicates that the means of MPCE over 5 animals will be distributed appropriately as a normal distribution. Thus parametric testing should be valid. One-tailed Student *t*-test analysis was performed to compare micronucleated polychromatic erythrocyte (MPCE) and PCE/NCE values between treated and control animals for significance. The Cochran-Armitage one-tailed trend test was used to analyze the dose response caused by each compound over all dose levels tested (Snedecor and Cochran, 1967).

TABLE 1

MICRONUCLEUS FORMATION AND PCE/NCE RATIOS IN BONE-MARROW CELLS OF MICE FOLLOWING EXPOSURE TO 4-CHLORO-*o*-PHENYLENEDIAMINE

Treatment (mg/kg)	Harvest time (h)	MPCE [a] ±S.D.	PCE/NCE [b] ±S.D.
Solvent control (0.2 ml DMSO)	24	1.9±1.02	1.1±0.61
Positive control (1.5 mg/kg TEM)	24	83.8±7.29	0.9±0.09
100	24 [c]	4.8±2.05 [e]	0.7±0.23
	48 [c]	1.7±0.91	1.0±0.17
	72 [d]	1.2±0.91	1.4±0.72
200	24 [c]	3.5±0.91	1.0±0.30
	48 [c]	1.3±1.19	0.7±0.12
	72 [d]	1.8±1.86	0.9±0.43
400	24 [c]	7.4±2.90 [e]	0.6±0.19
	48 [c]	9.0±9.08	0.8±0.32
	72 [d]	3.9±2.87	1.3±0.58

[a] Micronucleated polychromatic erythrocytes in 1000 PCE± standard deviation. Values represent means of 5 animals. 2000 cells were scored from each mouse.

[b] Ratio of polychromatic to normochromatic erythrocytes in the first 1000 PCE scored per animal. Values represent means of 5 animals.

[c] Sampling intervals of both 24 and 48 h were significant at a level of $p < 0.01$ in Cochran–Armitage trend test.

[d] Sampling interval of 72 h was significant at a level of $p < 0.05$ in the Cochran-Armitage trend test.

[e] Significant at $p < 0.05$ in a one-tailed *t*-test.

Results

COP induced significant dose-related increases in micronucleus frequencies. These increases occurred at all three sampling intervals (Table 1). The peak response was found at 24 h, where the highest dose tested, 400 mg/kg, induced 7.4 MPCE per 1000 PCE compared to 1.9 MPCE of the control. Values for PCE/NCE ratios in mice treated with COP were not significantly different from control values except for the highest treatment of 400 mg/kg when sampled at 24 h (Table 1). That ratio decreased from 1.1 (solvent control) to 0.6; a reduction of almost 50%.

Because of the low background MPCE frequency in the NOP experiment, a separate experiment was performed for the 24-h sampling time. The results from both experiments show that mice treated with NOP did not exhibit any dose-related response in micronuclei induction over the three dose levels tested when sampled at 24, 48 or 72 h (Table 2). Ratios of PCE to NCE in NOP-treated animals were not significantly different from the solvent control for all treatment levels and sampling times.

PPD did not cause a significant dose- or sampling time-related response in micronuclei induction (Table 3). Ratios of PCE/NCE in bone-marrow cells of mice exposed to PPD exhibited significant decreases at the highest dose 100 mg/kg tested at sampling times of 24 and 72 h (Table 3). Other significant decreases occurred at dose levels of 25 mg/kg at 48 h and 50 mg/kg at 72 h. The response was related to time and dose. The highest

TABLE 2

MICRONUCLEUS FORMATION AND PCE/NCE RATIOS IN BONE-MARROW CELLS OF MICE FOLLOWING EXPOSURE TO 4-NITRO-*o*-PHENYLENEDIAMINE

Treatment (mg/kg)	Harvest time (h)	MPCE [a] ± SD	PCE/NCE [b] ± SD
Positive control (1.5 mg/kg TEM)	24	80.1 ± 2.56	0.5 ± 0.02
Solvent control (0.2 ml DMSO) (24-h Expt.)	24	1.5 ± 0.71	0.8 ± 0.33
125	24	1.6 ± 0.49	0.7 ± 0.35
250	24	1.8 ± 0.14	0.8 ± 0.36
500	24	1.6 ± 0.74	0.8 ± 0.29
Solvent control (0.2 ml DMSO) (48- and 72-h Expt.)	24	0.4 ± 0.65	1.4 ± 0.35
125	48	0.2 ± 0.45	1.3 ± 0.07
250	48	0.9 ± 0.74	1.2 ± 0.05
500	48	0.2 ± 0.27	1.3 ± 0.06
125	72	0.4 ± 0.42	1.2 ± 0.10
250	72	0.7 ± 0.27	1.2 ± 0.13
500	72	0.4 ± 0.55	1.4 ± 0.23

[a] Micronucleated polychromatic erythrocytes in 1000 PCE ± standard deviation. Values represent means of 5 animals. 2000 cells were scored from each mouse.

[b] Ratio of polychromatic to normochromatic erythrocytes in the first 1000 PCE scored per animal. Values represent means of 5 animals.

dose showed decreases at two of the three sampling times. A sampling time of 72 h reduced the ratio in the highest two of the three dose levels administered.

Discussion

The micronucleus assay was used to examine the chromosomal damage effects of three structurally-related phenylenediamines. The number of micronuclei was found to be related to dose of COP. If the probability of a micronucleus being produced is linearly related to dose, then for any non-zero dose, the probability of producing a micronucleus would be different from that for solvent controls. However, examining the data indicates that the relationship may not be linear for the doses examined. In fact, comparing each treatment to the control (which is a weak test), results in a significant increase only for 100 and 400 mg/kg at time 24 h. The 24-h data are consistent with the possibility of a linear trend somewhere between 0 and 100 mg/kg and a constant effect for 100–400 mg/kg. The 48-h and 72-h data are consistent with the lack of an effect at doses of 200 mg/kg or less and perhaps a linear trend at higher doses. The highest response occurred at 24 h. Compounds usually cause a peak response in micronucleus induction and with a majority of compounds this peak occurs between 24 and 48 h after treatment (Heddle et al., 1983; MacGregor et al., 1987). COP was also able to affect more than one erythroblast cell cycle, thus causing increases at 48 and 72 h.

These results agree with other genotoxicity studies on COP. In the Ames Salmonella assay COP was a positive mutagen in strains TA98 and

TABLE 3

MICRONUCLEUS FORMATION AND PCE/NCE RATIOS IN BONE-MARROW CELLS OF MICE FOLLOWING EXPOSURE TO *p*-PHENYLENEDIAMINE DIHYDROCHLORIDE

Treatment (mg/kg)	Harvest time (h)	MPCE [a] ± SD	PCE/NCE [b] ± SD
Solvent control [c] (0.2 ml SDW)	24	1.3 ± 0.76	1.2 ± 0.17
Positive control (1.5 mg/kg TEM)	24	78.2 ± 5.40	0.3 ± 0.03
25	24	1.2 ± 0.76	1.3 ± 0.33
	48	1.2 ± 0.27	1.0 ± 0.22 [d]
	72	1.5 ± 1.00	1.2 ± 0.13
50	24	1.9 ± 0.65	1.2 ± 0.17
	48	2.0 ± 1.37	1.2 ± 0.22
	72	1.6 ± 0.74	0.9 ± 0.25 [d]
100	24	1.4 ± 1.11	0.8 ± 0.31 [e]
	48	1.5 ± 1.35	1.1 ± 0.36
	72	1.6 ± 1.03	0.9 ± 0.27 [d]

[a] Micronucleated polychromatic erythrocytes in 1000 PCE ± standard deviation. Values represent means of 10000 cells. 2000 cells were scored from each of 5 mice.

[b] Ratio of polychromatic to normochromatic erythrocytes in the first 1000 PCE scored per animal. Values represent means of 5 animals.

[c] Sterile distilled water (SDW).

[d] Significant at $p < 0.05$.

[e] Significant at $p < 0.01$.

TA100 with metabolic activation (Dunkel et al., 1985). Also, both with and without activation, COP caused DNA-strand breaks in human fibroblasts. However, the reactivity without activation was attributed to the human fibroblasts' intrinsic ability to activate. The study by McFee et al. (1986) showed that COP at the same doses tested as in this experiment (between 100 and 400 mg/kg) caused extensive damage to many of the chromosomes in 2–8% of the mouse bone-marrow cells but did not increase the percentage of aberrant cells. This could explain why the positive response in the micronucleus test was significant but not very strong. Even though a cell's chromosomes were extensively damaged only one MPCE would be scored in the micronucleus test.

Another reason for a weak response is that even though the compound was tested to levels which caused limited mortality, PCE/NCE ratios, an indicator of toxicity was only affected at the highest dose tested sampled at 24 h. This may indicate that the bone-marrow is not a major target organ for COP.

NOP did not cause an increase in MPCE. Earlier micronuclei studies of NOP have been inconclusive due to inadequate testing procedures (Heddle et al., 1983). Natarajan and Obe (1986) reported a negative response in bone-marrow micronuclei induction but a positive response in a liver micronucleus test. This difference was attributed to the fact that mutagens such as aromatic amines, for example NOP, which require metabolic activation are easily detected in the Ames assay with S9, whereas some of them may not be detected in the bone-marrow micronucleus test. This may be due to the inability of the active metabolites to reach the target cells in the bone-marrow whereas hepatocytes are directly involved in the activation of these compounds.

PPD did not induce micronculei at any of the doses or sampling intervals tested. There are no studies of PPD with the micronucleus test in the literature. However, PPD has been found to be mutagenic in the Ames assay in strains TA98 and TA100 with activation (Dunkel et al., 1985). Also, Shelby and Stasiewicz (1983) reported positive genotoxic activity in cultured mammalian cells with gene mutation, sister-chromatid exchange and chromosomal aberration assays. However, an in vivo study in the hepatocyte primary culture/DNA-repair test showed that PPD did not induce DNA damage (Williams et al., 1982). In this assay PPD was tested up to levels which exhibited limited mortality in the test animals. Also, PPD caused significant decreases in the PCE/NCE value at various treatments. This suggests that PPD actually reached the target cells in the bone-marrow, caused delay in the final erythroblast mitosis, but did not induce chromosomal damage detectable in the micronucleus assay.

The mouse bone-marrow micronucleus assay was able to distinguish the noncarcinogens from the carcinogen in this study. The results with COP and NOP in the study are similar to those reported by McFee and coworkers (1989). Other studies with the micronucleus assay and carcinogen/noncarcinogens have been performed. A report by Allen (1988) showed that in comparing micronucleus, sister-chromatid exchange and chromosome aberration in the testing of 2 carcinogen/noncarcinogen pairs that the micronucleus provided the most consistent clear positive results with carcinogens and negative results with presumptive noncarcinogens. In a study by Salamone et al. (1981) a success rate of ~60% in detecting carcinogens as positive and noncarcinogens as negatives was found. In addition, studies by Kirkhart (1981) and Tsuchimoto and Matter (1981) give lower success rates. It must be remembered that the micronucleus assay was developed to detect chromosome-breaking carcinogens and only will detect those where the target organ is the bone-marrow. Yet, overall the micronucleus assay is a most reliable one, if we keep these limitations in mind.

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