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Reproductive Effects of Alternative Disinfectants

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Organohalides formed through the reaction of chlorine and organic compounds in natural and waste waters pose potential health hazards. For this reason, alternative water disinfectants that do not form organohalides are being investigated with great interest. Limited data are available on the health effects, in particular reproductive toxicity effects, of these compounds.

In our laboratory, we have examined the reproductive effects of chloramine and chlorine administered by gavage in Long-Evans rats. Animals were treated for a total of 66 to 76 days. Males were treated for 56 days and females for 14 days prior to breeding and throughout the 10-day breeding period. Females were treated throughout gestation and lactation. Following breeding, the males were necropsied and evaluated for sperm parameters and reproductive tract histopathology. Adult females and some pups were necropsied at weaning on postnatal day 21. Other pups were treated postweaning until 28 or 40 days of age. These pups were evaluated for the day of vaginal patency and thyroid hormone levels.

No differences were observed between control rats and those rats exposed to up to 5 mg/kg/day chlorine or 10 mg/kg/day chloramine when fertility, viability, litter size, day of eye opening, or day of vaginal patency were evaluated. No alterations in sperm count, sperm direct progressive movement (μm/sec), percent motility, or sperm morphology were observed among adult male rats. In addition, male and female reproductive organ weights were comparable to their respective control groups, and no significant histopathologic changes were observed among chlorine- or chloramine-treated male and female rats.

Introduction

Chlorine has been the most commonly used disinfectant in the treatment of U.S. water supplies. However, potential health hazards are posed by organohalides formed through the reaction of chlorine with organic compounds present in natural and waste waters (*1, 2*). Trihalomethanes (THM), including chloroform, bromodichloromethane, and dibromochloromethane have been detected in chlorine-treated waters and are the subject of increasing concern regarding potential health effects. Additionally, chloroform has been designated as a carcinogen by the National Cancer Institute (*3*). The potential reproductive effects of chlorine and chlorine compounds have not yet been addressed fully, despite widespread use and the availability of a significant data base on the general toxicity of chlorine.

Druckrey (*4*) reported no effects on fertility, growth, blood parameters, survival, or the incidence of malignant tumors in seven consecutive generations of rats exposed to up to 100 ppm chlorine. Abdel-Rahman et al. (*5*) exposed rats to 1 to 100 ppm chlorine for 2.5 months before and throughout gestation. Pregnant rats were sacrificed on gestation day 20, and fetuses were examined for visceral and skeletal anomalies. No increase in fetal resorptions was observed in any dose group. Abdel-Rahman and co-workers reported a slight increase in skeletal variants among fetuses in the 10 and 100 ppm dose groups and some visceral anomalies in fetuses in the 100 ppm dose group. Unfortunately the number of litters per treatment group was small, so the fetus rather than the litter was used as the unit of comparison. A high percentage of control fetuses were also observed to have skeletal and visceral variants, reducing confidence in the apparent effect in this study.

Monochloramine (NH_2Cl) is currently used as an alternative to chlorine disinfection. Chloramine disinfection is advantageous because the persistence of chloramine provides a residual disinfectant in the water supply not maintained in chlorine-treated waters (*6*). Chloramine also provides the taste- and odor-control advantages of chlorine dioxide (ClO_2) use (*7*). Although NH_2Cl has been shown to be a weak mutagen (*8*), the only reported health effects associated with chloramine are hemolytic crises in long-term dialysis patients (*9,10*) and *in vitro* methemoglobin formation and hemolysis in human erythrocytes (*10*). Abdel-Rahman (*5*) reported no adverse effects in rat fetuses exposed *in utero* to up to 100 ppm NH_2Cl. Bercz et al. (*11*) demonstrated a significant decrease in serum thyroxine in African green

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Table 1. Reproductive performance of Long-Evans rats treated with 0, 1.0, 2.0, or 5.0 mg/kg aqueous chlorine.

	0 mg/kg	1.0 mg/kg	2.0 mg/kg	5.0 mg/kg
No. females bred	24	24	23	24
No. sperm positive	22	23	23	23
No. litters (D0)	19 (86%)	19 (83%)	22 (96%)	19 (83%)
No. litters (D4)	19 (100%)	18 (95%)	22 (100%)	19 (100%)
No. litters (D21)	19 (100%)	18 (95%)	22 (100%)	19 (100%)
Overall breeding success	79%	75%	96%	79%
$\bar{X}$ Litter size	13.3	13.3	13.4	12.8
Day of eye opening				
Median	Day 17	Day 16	Day 17	Day 17
Range	Days 15-20	Days 16-17	Days 17-19	Days 16-19

monkeys exposed to 100 ppm ClO_2, but observed no effects of chloramine on thyroid function. The studies reported here were designed to evaluate reproductive effects of chlorine and NH_2Cl in Long-Evans rats.

Materials and Methods

Long-Evans rats, 4–6 weeks of age, were obtained from Charles River Breeding Laboratories (Portage, MI) and were housed two per cage in polycarbonate cages with Absorb-Dri bedding. The animal room was maintained at 69–75°F and 40–60% humidity, with a 12-hr/12 hr light–dark cycle. Animals were given *ad libitum* access to Purina Certified Rodent Chow 5002 and deionized water. Two separate experiments were conducted. In the first study, the effect of aqueous chlorine on reproductive outcome was evaluated. Twelve male rats per dose group were gavaged with 0, 1.0, 2.0, or 5.0 mg/kg (10.0 mL/kg) aqueous chlorine (HOCl, pH 8.5) for 56 days prior to breeding and throughout the 10-day breeding period. Twenty-four female rats per dose group were given the same dose levels of chlorine by gavage for 14 days prior to breeding and throughout breeding, gestation, and lactation until the pups were weaned on day 21. Doses chosen were the highest practicable considering solution stability and potential gastric irritation. Animals were housed one male to two females throughout the 10-day breeding period. Females were checked for the presence of sperm in a lavage smear before 9:30 a.m. each morning. Females observed to be sperm positive (gestation day 0) were housed individually. The day of parturition was designated lactation day 0.

Following the breeding period, males were bled by

Table 2. Number and body weight of offspring exposed to aqueous chlorine.

Treatment		Day 1	Day 4	Day 21
Control (0 mg/kg)	Live	252	248	215
	Dead	0	2	0
	Culled	0	0	20[a]
	$\bar{X}$ Live/litter	13.3	13.0	10.3[b]
	$\bar{X}$ Body weight (g)	5.8	8.7	37.2
	N Litters	19	19	19
Chlorine (1 mg/kg)	Live	253	239	224
	Dead	0	14	0
	Culled	0	0	9[c]
	$\bar{X}$ Live/litter	13.3	13.3	11.9[c]
	$\bar{X}$ Body weight (g)	6.0	9.0	37.5
	N Litters	19	18	18
Chlorine (2 mg/kg)	Live	294	292	275
	Dead	3	2	2
	Culled	0	0	11[c]
	$\bar{X}$ Live/litter	13.4	13.3	12.0[c]
	$\bar{X}$ Body weight (g)	5.8	8.4	37.5
	N Litters	22	22	22
Chlorine (5 mg/kg)	Live	243	235	195
	Dead	0	2	6
	Culled	0	0	20[c]
	$\bar{X}$ Live/litter	12.8	12.4	9.2[c]
	$\bar{X}$ Body weight (g)	5.9	8.7	39.0
	N Litters	19	19	19

[a] Pups used for D-17 hormone bleeds.
[b] Number live - number culled/number litters.
[c] Pups used for practice hormone bleeds.

Table 3. Summary of male reproductive parameters in Long-Evans rats exposed to aqueous chlorine ($\bar{X}$ ± SD).

	0 mg/kg	1.0 mg/kg	2.0 mg/kg	5.0 mg/kg
No. males examined	12	12	11	11
Sperm conc. (X 10^9/g epidid.)	1.14 ± 0.22	1.03 ± 0.16	1.07 ± 0.13	1.06 ± 0.20
% Motility	18 ± 10	26 ± 12	20 ± 8	18 ± 17
Drive range (μm/sec)	7.9 ± 7.4	12.5 ± 5.4	11.0 ± 3.8	9.4 ± 7.0
% Abnormal morphology	0.90 ± 0.41	1.22 ± 0.54	0.71 ± 0.48	0.78 ± 0.33

Table 4. Reproductive performance of Long-Evans rats treated with 0, 2.5, 5.0, or 10.0 mg/kg aqueous monochloramine.

	0 mg/kg	2.5 mg/kg	5.0 mg/kg	10.0 mg/kg
No. females bred	24	24	24	24
No. sperm positive	20	24	20	21
No. litters (D0)	19 (95%)	17 (71%)	14 (70%)	20 (95%)
No. litters (D4)	19 (100%)	17 (100%)	14 (100%)	20 (100%)
No. litters (D21)	18 (95%)	17 (100%)	14 (100%)	19 (95%)
Overall breeding success	79%	71%	58%	83%
$\bar{X}$ Litter size	11.3	12.5	12.0	12.0

Table 5. Number and body weights of offspring exposed to aqueous monochloramine.

Treatment		Day 1	Day 4	Day 21
Control	Live	214	213	174
(0 mg/kg)	Dead	0	1	19
	Culled	0	1	20[a]
	$\bar{X}$ Live/litter	11.3	11.2	9.7
	$\bar{X}$ Body weight (g)	6.6	9.8	44.3
	N Litters	19	19	18
NH_2Cl	Live	212	206	182
(2.5 mg/kg)	Dead	0	6	4
	Culled	0	0	20[a]
	$\bar{X}$ Live/litter	12.5	12.1	10.7
	$\bar{X}$ Body weight (g)	6.1	9.3	42.9
	N Litters	17	17	17
NH_2Cl	Live	168	168	147
(5.0 mg/kg)	Dead	0	0	4
	Culled	0	0	20[a]
	$\bar{X}$ Live/litter	12.0	12.0	43.7
	$\bar{X}$ Body weight (g)	6.5	9.8	10.5
	N Litters	14	14	14
NH_2Cl	Live	240	239	200
(10.0 mg/kg)	Dead	0	1	19
	Culled	0	0	20[a]
	$\bar{X}$ Live/litter	12.0	12.0	10.5
	$\bar{X}$ Body weight (g)	6.4	9.7	43.0
	N Litters	20	20	19

[a] Pups used for D-17 hormone bleeds.

retro-orbital puncture for a complete blood count and from the vena cava for determinations of thyroid hormone levels. They were then sacrificed by pentobarbital overdose, and given a complete gross necropsy. The reproductive tract, including testis, epididymis, prostate, and seminal vesicles, was removed, weighed, and, except for the right cauda epididymis, preserved in neutral buffered formalin for histopathologic evaluation. The right cauda epididymis was weighed and a small sample of seminal fluid was expressed from the cut end of the vas deferens onto the edge of a clean scalpel blade. This fluid was mixed with two drops of phosphate buffered saline (PBS) with 0.1% glucose (pH 7.4, 37°C) on a prewarmed microscope slide. The slide was coverslipped and immediately observed using phase-contrast microscopy. The cauda was then finely minced in 10 mL of PBS. All slides, coverslips, solutions, and pipettes were maintained in an incubator at 37°C. A modification of Katz and Overstreet's (*12*) videomicrography method was used to videotape and evaluate a minimum of ten 10-sec fields at ×160 magnification and ten 10-sec fields at ×400 magnification. An aliquot of the minced cauda sperm suspension was heat-killed and used for determining sperm counts.

An 0.5 mL aliquot was heat-killed, stained with aqueous eosin Y, and used to prepare four to six slides

Table 6. Summary of male reproductive parameters in Long-Evans rats exposed to aqueous monochloramine ($\bar{X}$ ± SD).

	0 mg/kg	2.5 mg/kg	5.0 mg/kg	10.0 mg/kg
No. males examined	12	12	12	12
Sperm conc. ($\times 10^6$/g epidid.)	587.5 ± 155.8	602.0 ± 82.8	600.1 ± 202.6	644.6 ± 70.3
% Motility	27.8 ± 15.2	25.8 ± 12.2	24.7 ± 15.1	30.3 ± 10.5
Drive range (μm/sec)	19.67 ± 11.95	17.38 ± 8.35	11.95 ± 9.38	13.17 ± 9.02
% Abnormal morphology	1.1 ± 1.3	0.5 ± 0.4	2.6 ± 4.7	0.8 ± 0.5

per animal for sperm morphology evaluation. The videotapes were later evaluated for percent sperm motility (10 fields) and sperm direct progressive movement (five sperm/field; ten fields). Sperm motility was estimated by two independent observers. Sperm showing any movement were judged motile. The observers' results agreed within 5%. Sperm for progressive movement determinations were randomly selected with the videotape stopped. The tape was advanced, and the distance traveled was measured. The distance traveled on the videoscreen (millimeters) was correlated with the actual distance traveled (micrometers). The elapsed time was determined from a running digital time clock that was videotaped simultaneously when videotaping the sperm. Only morphologically normal sperm were selected for progressive movement determinations.

Dams were observed for fertility, length of gestation, body weight gain, and maternal behavior. At necropsy on lactation day 21, dams were bled by retro-orbital puncture for complete blood counts and via the vena cava for hormone analysis, then sacrificed by pentobarbital overdose and given a complete gross necropsy. The reproductive tract was removed, weighed, and preserved for histopathologic evaluation.

Litters were evaluated for viability, litter size, day of eye opening, body weight gain, and gross external abnormalities. The day on which all pups in a litter had both eyes open was designated as the day of eye opening. At necropsy on lactation day 21, 10 pups/sex/dose were bled for complete blood counts and hormone analysis. The reproductive tract was weighed and preserved. Randomly selected pups were retained for observation of the day of vaginal patency and hormone evaluation.

In the second experiment, groups of 12 male and 24 female rats were administered 0, 2.5, 5.0, or 10.0 mg/kg (10.0 mL/kg) chloramine in deionized water by oral gavage for 66 to 76 days according to the regimen described for the first experiment. Chloramine stock solution was prepared by titrating chlorine stock solution with ammonia. All other methods were the same as that described for the study of chlorine.

Results

Chlorine

Neither clinical signs of toxicity, hematology changes, nor body weight depression were observed for male or female Long-Evans rats exposed to 0, 1.0, 2.0, or 5.0 mg/kg chlorine in deionized water for up to 76 days. The observed fertility rate varied between 75% and 96%, but did not vary in a dose-dependent manner (Table 1). Litter survival, litter size, and pup weight did not differ among groups (Table 2). Developmental landmarks such as the mean day of eye opening (day 17) and average day of observed vaginal patency (day 33) were comparable across groups. The day of eye opening is a general maturational index, whereas the day of vaginal patency provides information on rate of sexual maturation. Adult male rats exposed to up to 5.0 mg/kg HOCl showed no adverse reproductive effects (Table 3). Sperm concentration ranged from 1.03×10^9 sperm/g epididymal tissue (1.0 mg/kg HOCl dose group) to 1.14×10^9 sperm/g epididymal tissue (control group). The percentage of motile sperm and sperm progressive movement (μm/sec) were not different when compared to controls. The percentage of abnormal sperm morphology was comparable for all groups, ranging from 0.71% (2.0 mg/kg HOCl dose group) to 1.22% (1.0 mg/kg HOCl dose group). No histopathologic lesions were observed in the reproductive tract of adult male or female rats exposed to 0 to 5.0 mg/kg chlorine.

Chloramine

No clinical signs of toxicity, hematology changes, or evidence of body weight suppression were observed for adult male or female rats dosed with 0, 2.5, 5.0, or 10.0 mg/kg NH_2Cl by gavage for up to 73 days. Fertility, fecundity, length of gestation, litter size, and litter survival rates were unchanged by NH_2Cl treatment (Table 4). Mean pup weight also was unaltered (Table 5). The average day of eye opening (day 16) and the average day of observed vaginal patency (day 31.8 to day 32.6) were comparable among groups. No treatment-related adverse reproductive effects were observed in adult male rats treated with up to 10.0 mg/kg NH_2Cl. These results are presented in Table 6. The sperm concentration ranged from 587.5×10^6 sperm/g epididymal tissue to 644×10^6 sperm/g epididymal tissue. The percentage of abnormal sperm morphology ranged from 0.4 ± 0.5% to 2.6 ± 4.7%, but did not vary in a dose-dependent manner. Sperm motility and sperm progressive movement were unaffected by dose exposure. No treatment-related histopathologic lesions were observed in the reproductive tracts of male or female rats.

Discussion

There is a dearth of reproductive and developmental toxicity data for chlorine and alternative water disinfectants (e.g., chloramine, chlorine dioxide) and their

by-products such as chlorite and chlorate. Evaluations of chlorine and NH_2Cl (5) have indicated that these chemicals have little or no teratogenic effect. A slight increase in skeletal variants and soft tissue variations were noted, but the number of dams per group for both chemicals were insufficient to evaluate teratogenicity. Druckrey (4) reported no toxicity associated with exposure of seven consecutive generations of rats to up to 100 ppm chlorine.

Our studies of the potential reproductive toxicity of chlorine and chloramine also indicate no adverse reproductive effects following subchronic administration of these two drinking water disinfectants. Although the percentage of abnormal sperm forms in the 5.0 mg/kg NH_2Cl dose group (2.6 ± 4.7%) was elevated relative to control males (1.1 ± 1.3%), the large standard deviation of the former precluded statistical significance. Abnormal sperm were observed in only two of the 12 rats at 5.0 mg/kg, and no adverse effects were observed among males treated with 10.0 mg/kg NH_2Cl. Thus, this result was not considered to be chloramine-induced.

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REFERENCES

1. Bellar, T. A., Lichtenberg, J. J., and Kroner, R. D. The occurrence of organohalides in chlorinated drinking waters. J. Am. Water Works Assoc. 66: 703–706 (1974).
2. Rook, J. J. Formation of haloforms during chlorination of natural waters. J. Water Treatment Exam. 23: 234–236 (1974).
3. National Cancer Institute. Report on the Carcinogenesis Bioassay of Chloroform. NCI, Bethesda, MD, 1976.
4. Druckrey, H. Chloriertes Trinkwasser, Toxizitats-Prüfungen an Ratten uber sieben Generationen. Food Cosmet. Toxicol. 6: 147–154 (1968).
5. Abdel-Rahman, M. S., Buardi, M. R., and Bull, R. J. Effect of chlorine and monochloramine in drinking water on the developing rat fetus. J. Appl. Toxicol. 2: 156–159 (1982).
6. Durfor, C. N., and Becker, E. Public Water Supplies of the 100 Largest Cities in the United States. USGS Water Supply Paper 1812, 1962.
7. Brodtmann, N. W., Jr., and Russo, P. J. The use of chloramine for reduction of trihalomethanes and disinfection of drinking water. J. Am. Water Works Assoc. 71: 40–42 (1979).
8. Shih, K., and Lederberg, J. Chloramine mutagenesis in *Bacillus subtilis*. Science 192: 1141–1143 (1976).
9. Kjellstrand, C. M., Eaton, J. W., Yawata, Y., Swofford, H., Koplin, C. F., Buselmeier, T. J., Hartitizsch, J., and Jacob, H. Hemolysis in dialyzed patients caused by chloramines. Nephron 13: 427–433 (1974).
10. Eaton, J. W., Dolpin, C. F., Kjellstrand, C. M., and Jacob, H. S. Chlorinated urban water: a cause of dialysis-induced hemolytic anemia. Science 181: 463–464 (1973).
11. Bercz, J. P., Jones, L., Garner, L., Murray, D., Ludwig, D. A., and Boston, J. Subchronic toxicity of chlorine dioxide and related compounds in drinking water in the nonhuman primate. Environ. Health Perspect. 46: 47–55 (1982).
12. Katz, D. F., and Overstreet, J. W. Sperm motility assessment by videomicrography. Fertil. Steril. 35: 188–193 (1981).