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Chronic Exposure to Low Concentrations of Halothane–Nitrous Oxide:

Reproductive and Cytogenetic Effects in the Rat

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To determine whether prolonged exposure to low-concentration combinations of halothane and nitrous oxide would adversely affect fertility, gestation, or fetal development or damage chromosomes *in vivo*, adult male and female rats were exposed for 60 days prior to mating either to halothane, 1 ppm, and nitrous oxide (N_2O), 50 ppm, or to these agents at 10 and 500 ppm, respectively, for seven hours/day, five days/week. Inseminated females were either exposed seven hours/day from Day 1 through Day 15 or from Day 6 through Day 15 of their putative gestation. The former were allowed to deliver normally; fetuses of the latter were delivered by cesarean section on Day 10. The males were exposed for an additional 40 weeks (after the three-week mating period), after which bone marrow and spermatogonial cells were harvested for cytogenetic analysis.

Exposure to halothane, 10 ppm, plus N_2O, 500 ppm, resulted in decreased ovulation and implantation efficiency and in slightly retarded fetal development (also seen at the lower level exposure). No major teratologic effect or unequivocal abortifacient effect of exposure of pregnant females during organogenesis or prior exposure of males was observed. However, cytogenetic damage to both bone marrow and spermatogonial cells was found at both levels of exposure. (Key words: Anesthetics, gases: nitrous oxide. Anesthetics, volatile: halothane. Toxicity: teratogenicity; mutagenicity; trace concentrations.)

SOME EPIDEMIOLOGIC REPORTS have suggested that occupational exposure to conditions in operating rooms may be associated with higher than normal incidences of spontaneous abortions[1–5] and congenital abnormalities in the offspring of those conceiving.[6] A common denominator in all these reports was that the operating room personnel had been repeatedly exposed to low levels of volatile anesthetics in the course of their daily occupation. Halothane is the suspected toxic agent, although the possibility of potentiation by nitrous oxide is not without foundation.[7]

Animal studies with low levels of halothane alone have not consistently shown reproductive effects. Doenicke *et al.*[8] exposed rats daily to 8,000 ppm and found a very low birth rate, whereas Bruce[9] and Wharton *et al.*[10] found no such effect in mice exposed to 16 ppm and 500 ppm, respectively. For the most part, animal exposures have been to levels of halothane or nitrous oxide well above 10 ppm and 500 ppm, respectively, and for relatively short periods of time except in relation to gestation periods in studies of pregnant rodents. It is likely that prolonged exposure to halothane at very low levels (*e.g.*, 1–10 ppm) results in chronic systemic exposure to its metabolites, which is not paralleled by acute exposure to high levels, which saturate the metabolic enzyme system.[11] To simulate the exposures of operating room personnel, exposures of experimental female animals should precede mating by a considerable period, as well as occur during pregnancy. Similarly, males should be subjected to chronic exposure prior to mating. It was the purpose of the experiments reported herein to investigate reproductive, teratogenic, and mutagenic (cytogenetic) effects in rats exposed chronically to combinations of halothane and nitrous oxide in the range of concentrations encountered in operating rooms not equipped with gas-scavenging devices.[12,13] Specifically, we wished to examine the rats for these effects from prolonged exposures to halothane, 10 ppm, combined with nitrous oxide, 500 ppm, and to halothane, 1.0 ppm, plus N_2O, 50 ppm.

Materials and Methods

Young sexually mature cesarean section-derived and barrier-sustained Sprague-Dawley rats,§ 120 males and 120 females, were divided randomly into three equal groups. For seven hours/day, five days/week, for 12 weeks, one group of 40 male and 40 female rats (Group I) was exposed to filtered air; Group II was exposed to halothane, 1.2 (±0.3) ppm, plus N_2O, 48.8 (±5.8) ppm; Group III was exposed to halothane, 11.8 (±2.5) ppm, plus N_2O, 499.1 (±17.9) ppm.

All animals lived continuously (except for daily housekeeping) in glass and stainless steel exposure chambers 6 cu m in size, operated with 1.2 cu m/min filtered airflow. The animals were housed in stainless

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steel wire baskets, five per sex per basket, on a single tier at the midline of the chamber. Top-loading feeders and water bottles allowed eating and drinking *ad libitum*. After exposures for seven hours/day, five days/week, for 12 weeks, the animals were removed for breeding. Subsequent exposures are described below. The animals were on a fixed diurnal cycle of 12 hours of light and 12 hours of darkness with exposures during the light phase.

The HEPA-filtered air input of the chambers was via a duct tangential to the cylindrical turret at the apex of a pyramidal roof, and the exhaust was via a goose-necked duct at the center of the inverted pyramidal bottom. A single-pass system was in effect. N_2O was supplied from pressurized cylinders containing certified 98 per cent pure nitrous oxide anhydride. ¶The gas was metered with a glass flowmeter and critical orifice into a three-necked glass mixing flask prior to its insertion into the chamber air input duct. A few ml/min of nitrogen ¶(99.8 per cent) were passed via a flowmeter and critical orifice through the headspace of a brown glass flask containing liquid halothane** and then into the three-necked mixing flask to mix with the N_2O prior to insertion into the chamber input duct. The chambers were operated under negative pressure. The chamber airflow was monitored by low-pressure vacuum gauges with limit switches†† controlling normally-closed solenoid valves on the N_2O and nitrogen cylinders to prevent overexposures due to possible downward fluctuations in air flow.

The concentrations of halothane and N_2O in each chamber were determined from a sample line continuously functioning at 10 l/min from a Teflon® probe located just about the center cage. Halothane was measured by peak-height analysis on a gas chromatograph equipped with an electron capture detector. Gas samples were taken by gas-tight syringe from a septum in the sample line, injected into a Vacutainer® containing a fixed volume of 2,2,4-trimethyl pentane (TMP), and then a 1.0-ml volume of the latter was injected into the gas chromatograph. Halothane was measured at least daily and more often, four times per day. Liquid halothane-in-TMP standards were prepared daily. Nitrous oxide was monitored continuously on a LIRA® infrared spectrometer validated by gas chromatography.[14] Hourly readings were recorded.

Following the 12-week exposure period, animals within the respective groups were allowed to mate randomly by placing single males with single females in mesh cages outside the chamber until spermatozoa were detected by microscopic inspection of a daily saline vaginal douche made early in the morning. Uninseminated females were rotated to proven males after seven and 14 days. On the day spermatozoa were observed (designated Day 0 of gestation), the female animals were housed in sex-segregated cages by groups and the males either rotated to new females or similarly housed. The total span of breeding effort was 21 days for any one animal.

The first 20 mated females (*i.e.*, those in which spermatozoa were observed) were to be used for evaluating teratologic effects and were designated for cesarean-section delivery of the fetuses. These animals were re-exposed to their respective atmospheres during the period of major organogenesis, *i.e.*, from Day 6 through Day 15 of gestation. The remaining mated females in each group were to be used for evaluation of effects on reproduction and were designated for natural delivery. They were re-exposed to their respective atmospheres from Day 1 through Day 15 of gestation. Exposure levels were within 10 per cent of the target concentrations. Exposures were again for seven hours/day. Unmated females were discarded.

Following the 21-day mating period, all males were returned to their respective exposure atmospheres and exposed for seven hours/day, five days/week for 40 additional weeks. Exposure levels remained within 10 per cent of the target concentrations. Throughout the exposure and mating periods, all animals were observed daily for mortality and other indications of toxicity. The females were weighed on gestation Days 0, 5, 16, and 20.

The 20 mated females per group re-exposed from Day 6 through Day 15 of gestation were sacrificed on Day 20 by chloroform overdosage. Cesarean sections were performed. The number of corpora lutea, the number of and placement of uterine implantation sites, the number and placement of live and dead fetuses, individual fetal weights, individual fetal crown–rump lengths, and external fetal abnormalities were recorded. The uterus and ovaries of each adult female were examined and abnormalities recorded. Approximately a third of the fetuses from each pregnant female were fixed in Bouin's solution and examined for visceral abnormalities by the method of Wilson and Warkany.[15] Whole-body transverse sections of the nasal, orbital, cervical, thoracic, and abdominal regions were examined under a dissecting microscope.

The remaining two thirds of the fetuses were prepared for skeletal examination by fixation in 95 per cent ethanol for 72 hours, followed by 24 hours in 2

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** Ayerst Pharmaceutical Co., New York, N. Y.

†† Photohelic Pressure Switch, Dwyer Manufacturing Co., Michigan City, Ind.

per cent potassium hydroxide, and then stained for 24 hours in a solution of alizarin red S and KOH and rinsed in distilled water. The stain was extracted from the soft tissue by soaking the fetuses for 24 hours in a solution of one part each benzyl alcohol and glycerin with two parts 70 per cent ethanol. The fetuses were then cleared with 75 per cent aqueous glycerin for 24 hours. Lamps were kept over the specimen containers throughout staining and clearing procedures. Each skeleton was evaluated for relative size differences, locations of normal or abnormal bone structures, extent of ossification, and the presence of extra or missing bone structures in the skull, ribs, sternebrae, vertebrae, pelvis, forepaws, and hindpaws.

The remaining mated females in each group, which were re-exposed to treatment from Day 1 through Day 15 of gestation, were then removed from the exposure chambers and allowed to deliver normally and carry their litters to weaning at three weeks. Within 24 hours following birth, the litters were examined for the number and sex distribution of live and dead pups, the litter weight by sex recorded, and the pups were examined for evidence of external anatomic abnormalities. The litters were arbitrarily decreased to a maximum of eight pups (equally divided by sex when possible) to be carried through a 21-day lactation period to weaning. Litter weights by sex were recorded again four days post partum and at weaning (21 days post partum). Mortality, appearance, and behavior of the offspring were also recorded at these intervals during lactation. At weaning (Day 21), all surviving maternal females were sacrificed and gross necropsies were performed; these included examination of the viscera and recording of the number of ovarian corpora lutea and evidence of implantation sites. Necropsies were also performed on any females that died and on all surviving pups at weaning.

The male rats that survived a total of 52 weeks of exposure, interrupted by the 21-day mating period, were sacrificed during chloroform anesthesia by cervical dislocation five hours after administration of colchicine, 2 mg/kg, *ip*, in distilled water. Immediately following death, bone marrow cells were collected from both femurs of each animal and the testes were excised and spermatogonial cells processed.

Bone marrow cells were harvested by aspiration of marrow into prewarmed (37 C) Hanks' balanced solution, and the cells were treated with hypotonic KCl (0.055 M) for 30 min. After fixation in a 3:1 methanol–acetic acid fixative for at least 60 min, the cells were centrifuged and resuspended in fresh fixative. Three or four drops of this suspension were placed on a glass slide, which was passed through a flame until dried. Slides were code-numbered without reference to test groups.

Spermatogonial cells were obtained by removing the tunica albuginea and thoroughly mincing the seminiferous tubules. The minced tubules were suspended in 10 ml of prewarmed (37 C) hypotonic sodium citrate (1 per cent). The suspension was then transferred to a 12-ml test tube and allowed to settle. The supernatant was discarded and the remaining tubules resuspended in 10 ml of fresh 1 per cent sodium citrate. After 8 min, the supernatant was pipetted out and the tubules fixed in 3:1 methanol–acetic acid for 24 hours at 4 C. The fixed minced tubules were then centrifuged at 1,000 rpm for 10 min, the fixative discarded, and the tubules exposed to 5 ml of 60 per cent acetic acid for at least 3 min. The test tube was gently agitated to produce a homogeneous cell suspension and then 0.2 ml of the suspension was removed with a Pasteur pipette and placed on a prewarmed (≈50 C) slide on a hotplate. The liquid was immediately withdrawn from the slide. This was repeated until the slide was covered with small areas of dried cells. The slides were then code-numbered without reference to test groups.

At least three bone marrow and three spermatogonial cell slides were prepared for each animal using Giemsa stain. Twenty-five metaphases were evaluated microscopically from each preparation that was analyzable and the vernier location recorded for each metaphase. Chromatid breaks (involving one chromosomal arm) were scored as either with or without a visible fragment. All markers, including exchanges, dicentrics, rings, and other abnormal chromosomes, were scored separately. Where more than one type of aberration was observed in any one metaphase, the cell was scored as having two or more aberration types. Those cells with more than nine aberrations (whether of one or more types) were scored as having ten or more aberrations. Unique markers in these cells were tabulated and an estimate of polyploidy was made for each cell at the time of evaluation. Representative photographs showing characteristic aberrations as well a normal cells were made of all slides. Karyotypic analysis was not undertaken. In addition, the numbers of gaps (regions of chromatid arms with stained areas less than the width of the chromatid arm) were scored but not used in any calculations. All slides were scored blindly with no reference to group identification. Upon completion of all scoring, the data sheets were placed into the appropriate groups and statistical analyses were performed. The bone marrow slides were completed prior to beginning the evaluation of spermatogonial cells.

Each category of aberrations was recorded in two ways: 1) the number of aberrations/animal, and 2) the number of cells showing a particular aberration/animal. Thus, it could be determined how widespread each type of effect observed was within each animal as against the number of effects within the animal. Unless otherwise indicated, all analyses were made comparing the mean number of affected cells or the mean number of aberrations for each treatment group with the corresponding mean for the control group by analysis of variance (F test) and by the Student *t* test.‡‡ When variances differed significantly, the Student *t* test was appropriately modified (t') and Cochran's approximation utilized.[16] Terminal body weights were similarly analyzed. Finally, the proportion of animals showing aberrations and the proportions of examined cells showing aberrations in each group were analyzed by the chi-square test. The level of probability chosen for rejecting the null hypotheses was 0.05.

Results

Of the original 120 female rats, one Group II rat died during Week 8 of exposure and two Group III rats scheduled for natural delivery died during the post-mating period. One of these later was found not to be pregnant, despite the observation of vaginal spermatozoa during the mating period. The other died with her pups unborn. Of the original 120 males, all survived the pre-mating exposure and 114 survived the 52-week exposure: 39/40 in Group I, 37/40 in Group II, and 38/40 in Group III. Thus, no treatment effect on survival was observed.

The group mean body weights of the females were nearly identical at Day 0 of gestation. However, on Day 20 of gestation, the mean body weights of pregnant females in Groups II and III were significantly lower than that of pregnant females in Group I. A similar, but nonsignificant, difference was observed among those found not to be pregnant in the natural-delivery subgroups. No significant difference was seen at Day 20 among those scheduled for cesarean section (exposed during Days 6–15 of gestation). There was no significant difference among the males at termination.

The fertility indices (number of females pregnant§§/number of females mated¶¶ 100) for the females designated for cesarean section were 65 (13/20), 80 (16/20), and 65 per cent (13/20) for Groups I, II, and III, respectively. The fertility indices for the females scheduled for natural delivery were 89 (17/19). 72 (13/18), and 37 per cent (7/19) for Groups I, II, and III, respectively. The fertility index for Group III was significantly lower than the index for the natural-delivery controls ($\chi^2 = 11.31$, d.f. = 1) and the overall Group III fertility index was significantly lower than that of the controls ($\chi^2 = 5.57$, d.f. = 1). With the exceptions of significantly lower mean fetal weight and mean fetal length for Group III, all values for the ovarian, uterine, and litter data, as well as the indices of pre-implantation loss, implantation efficiency, and post-implantation loss, were similar in all three groups of females used for cesarean section (table 1). Findings at necropsy included a number of incidental changes that were considered unrelated to treatment and were observed at comparable incidences in the control and treated groups.

Gross examination of fetuses for external anatomic abnormalities and soft-tissue examinations by Wilson's technique revealed no consistent unusual finding among the test or control fetuses. Major malformations consisted of ectopia of the right kidney in one fetus in Group I. Fetal skeletal evaluation revealed slight retardation of skeletal development in Groups II and III when compared with Group I. This retardation was indicated by significantly higher percentage incidences of non-ossification in the sixth sternebrae, 25th–26th caudal vertebrae, 3rd–4th metacarpals of the forepaws, and 24th–28th phalanges of the forepaws for the members of Groups II and III. No dose-response relationship was observed.

Among the natural-delivery females the mean numbers of ovarian corpora lutea for Groups II and III and the number of uterine implantation sites for Group III were significantly lower than corresponding values obtained for the Group I control females (table 2). Implantation efficiency for Group III was significantly lower than implantation efficiency for the control group ($\chi^2 = 41.44$, d.f. = 1). A chi-square analysis of the percentage of implants not accounted for in dead fetuses or live births observed showed that Group III had a significantly larger proportion unaccounted for than Group I ($\chi^2 = 5.13$, d.f. = 1). Either these were resorbed or the pups were cannibalized at birth. The "effect" was largely due to the complete loss of 13 implantations in one of the seven pregnant females in Group III. Since one female in Group I also lost 13 of 13 implantations, it is not clear that a compound effect should be inferred.

Only one instance of dystocia or delayed or prolonged labor was observed among the control and test

‡‡ Two-tailed tests.

§§ Diagnosis of pregnancy was based on the presence of uterine implantation sites.

¶¶ Mated females were those in which spermatozoa were observed in vaginal washings.

TABLE 1. Cesarean Delivery: Group Mean Ovarian, Uterine, and Litter Data (±SD) and Group Means for Reproductive Indices for Pregnant Females

	Group I	Group II	Group III
Ovarian corpora lutea	18.5 ± 3.1	15.2 ± 3.2	14.8 ± 2.3
Uterine implantation sites	13.4 ± 1.7	11.9 ± 3.4	11.9 ± 3.0
Resorption sites	0.5 ± .8	0.8 ± .7	0.8 ± 1.2
Live fetuses	12.8 ± 1.9	11.1 ± 3.5	11.2 ± 2.7
Dead fetuses	0	.06 ± .30	0
Fetal weight (g)	4.1 ± .25	4.0 ± .66	3.7* ± .43
Fetal length (cm)	4.1 ± .1	4.0 ± .2	3.9* ± .2
Implantation efficiency (per cent)	72	78	80
Post-implantation loss index (per cent)	4	7	8

* Significantly lower than corresponding value for cesarean-delivery controls, $P < 0.05$.

animals allowed to complete the gestation period with natural deliveries. One animal in Group III was found dead on Day 24 of gestation with one dead fetus in the vagina and 13 dead fetuses in the uterine horns. The gestation index (number of full-term litters/numbers of pregnancies) for Group III was significantly lower than the index for the control group ($\chi^2 = 4.88$, d.f. = 1). Mean durations of the gestation periods were 22.7, 23.1, and 21.8 days for Groups I, II, and III, respectively (excluding the one Group III pregnant female found dead on Day 24 of gestation).

There was no indication of exposure-related effect with regard to the incidence of stillbirths or the incidence of pups found dead or missing during lactation. One Group I, five Group II, and two Group III pups were found dead within 24 hours of delivery. Fourteen Group I, 14 Group II, and one Group III pup were either found dead or found to be missing due to apparent cannibilization during lactation. Live births and lactation indices were similar among the groups. There was no indication of a treatment-related effect regarding the mean numbers of male and female offspring produced per litter in either of the test groups. The appearance and behavior of offspring of the treated females revealed no evidence of exposure-related effect, although the pups from the high-anesthetic concentration group were smaller than the controls at weaning.

Statistical analysis of group mean body weights of male and female offspring revealed no significant difference in either sex between the control and treated groups 24 hours and four days after delivery. At weaning, however, a significantly lower mean body weight was found for the Group III male pups when compared with the controls. The females were smaller but not significantly so. Gross necropsy of females that delivered naturally and of their offspring did not reveal any alteration in any organ or tissue attributable to the treatment program.

TABLE 2. Natural Delivery: Group Mean Ovarian and Uterine, and Litter Data (±SD) and Group Means for Reproductive Indices for Mated Females

	Group I	Group II	Group III
Ovarian corpora lutea	19.5 ± 3.8	15.4* ± 5.9	13.2* ± 5.2
Uterine implantation sites	10.9 ± 5.5	7.5 ± 6.2	3.8* ± 6.2
Implantation efficiency (per cent)	56	49	29*
Post-implantation loss index (per cent)	14	15	28*
Number of pregnancies (total)	17	13	7*
Number of full-term litters born (total)	16	12	4*
Gestation index (per cent)	94	92	57*
Number of pups born (total)	178	115	46*
Number of pups born alive (total)	177	110	44*
Live birth index (per cent)	99	96	96
Mean litter size	11	10	12
Number of pups left to nurse (total)	114	79	31
Number of pups weaned (total)	100	65	30
Lactation index (per cent)	88	82	97
Pup body weight (g) at 24 hours			
Males	6.6 ± .9	6.5 ± 1.2	6.1 ± 1.1
Females	6.0 ± .7	5.8 ± 1.1	5.6 ± 1.3
Pup body weight (g) at 4 days			
Males	9.9 ± 3.2	10.3 ± 1.8	9.7 ± 1.2
Females	9.5 ± 3.0	9.9 ± 1.5	9.2 ± 1.9
Pup body weight (g) at weaning			
Males	49.5 ± 8.2	50.4 ± 4.3	40.9* ± 5.5
Females	47.1 ± 6.7	44.8 ± 8.0	41.4 ± 3.4

* Significantly lower than corresponding value for natural-delivery controls, $P < 0.05$.

TABLE 3. Number of Rats and Cells with Aberrations (Excluding Gaps) in Chromosomal Structure

	Number of Rats Examined	Animals with Aberrant Cells		No. of cells Examined	Cells with Aberrations	
		Number	Per Cent		Number	Per Cent
Bone marrow						
Group I (control)	38	11	29	950	15	1.2
Group II	37	22*	60	925	30*	3
Group III	38	26*	68	950	50*	5
Spermatogonial						
Group I (control)	35	17	49	875	23	2.3
Group II	35	27*	77	875	59*	6.7
Group III	30	26*	87	750	95*	13

* Significantly higher than control value, $P < 0.05$.

There were 39 surviving males to be evaluated cytogenetically in Group I. One bone marrow slide was eliminated from Group I for statistical analysis since only 11 metaphases were analyzable, and four spermatogonial slides were similarly eliminated. Hence, data presented for Group I are based on 38 and 35 rats, representing a total of 950 bone marrow cells and 875 spermatogonial cells, respectively. In the same way, bone marrow and spermatogonial data presented for Group II were collected from 37 and 35 rats, representing 925 bone marrow and 875 spermatogonial cells, respectively. Data for Group III are from 38 and 30 rats, representing 950 bone marrow and 750 spermatogonial cells, respectively.

The mitotic indices were low in both Group II and Group III relative to Group I. Although inclusion of gaps would have increased the relative differences between groups, gaps were not considered cytogenetically important, so they were excluded from the evaluations.

Group I manifested a relatively high background frequency of aberrant cells in both bone marrow and spermatogonial cells (table 3). This was attributed to the age of the rats at termination (63 weeks). However, both exposure groups had significantly higher proportions of aberrant cells than the control group. The mean number of cells showing all types of aberrations (excluding gaps) for Groups II and III was significantly higher than the value obtained for the control group (table 4). Dose-related effects on the number of animals showing aberrant cells, the total number of cells showing aberrations, the mean number of aberrant cells, and the mean number of all types of aberrations were observed.

No significant difference was found for the bone marrow cells among the groups, but spermatogonial cells that had ten or more aberrations were significantly more frequent in Groups II and III when compared with the controls (table 5). The mean numbers of spermatogonial cells with two or more aberration types in Groups II and III were also significantly greater than the control value.

The mean number of cells with marker chromosomes in bone marrow cells in Group III was significantly higher than the value obtained for the control group (table 6). Of the 950 cells analyzed in the control group, six cells (0.6 per cent) had markers. Five of these cells had a total of ten exchange figures and one cell contained one miscellaneous metacentric marker. In Group II, 12 cells of the 925 (1.3 per cent) analyzed had a total of 18 exchange figures. Rings and metacentric markers were not observed in Group II. Statistically, this group was comparable to the control group. In Group III, however, there were 28 cells (3 per cent) which contained a total of 50 marker-type aberrations.

The mean numbers of spermatogonial cells showing markers in both low- and high-anesthetic-concentration groups were significantly increased above the number in Group I. Of the 875 cells in the Group I examined, 10 (1 per cent) manifested 12 marker chromosomes. All of these markers were exchanges.

TABLE 4. Mean Numbers ±SD of Analyzed Rat Cells per Animal Showing Any Aberration (Excluding Gaps)

	Bone Marrow	Spermatogonial
Group I (control)	0.39 ± 0.72	0.66 ± 0.76
Group II	0.81 ± 0.88*	1.69 ± 1.39*
Group III	1.32 ± 1.32*	3.17 ± 1.84*

* Significantly higher than control value $P < 0.05$.

TABLE 5. Mean Numbers ±SD of All Types of Aberrations (Excluding Gaps) per Animal

	Bone Marrow Cells	Spermatogonial Cells
Group I (control)	0.74 ± 1.69	0.69 ± 1.16
Group II	1.14 ± 1.36	2.89 ± 2.70*
Group III	2.37 ± 3.13*	6.63 ± 4.71*

* Significantly higher than control value, $P < 0.05$.

TABLE 6. Mean Numbers of Cells per Animal Showing Ten or More Aberrations and/or Cells with Two or More Types of Aberrations

	Mean Number of Cells with Ten Aberrations ±SD	Mean Number of Cells with Two or More Aberrations ±SD
Bone marrow		
Group I (control)	0.03 ± 0.16	0.11 ± 0.31
Group II	0.03 ± 0.16	0.19 ± 0.40
Group III	0.05 ± 0.23	0.34 ± 0.63
Spermatogonial		
Group I (control)	0.03 ± 0.17	0.09 ± 0.28
Group II	0.26 ± 0.51	0.49 ± 0.61*
Group III	0.73 ± 0.94*	1.30 ± 1.06*

* Significantly higher than control value, $P < 0.05$.

TABLE 7. Mean Numbers of Analyzed Cells per Animal showing Marker Chromosomes (Exchange Figures, Rings and Miscellaneous Markers)

	Mean Number of Bone Marrow Cells with Markers ±SD	Mean Number of Spermatogonial Cells with Markers ±SD
Group I (control)	0.16 ± 0.44	0.29 ± 0.52
Group II	0.32 ± 0.63	1.06 ± 1.19*
Group III	0.76 ± 1.17*	2.57 ± 1.72*

* Significantly higher than control value, $P < 0.05$.

In Group II, 37 of 875 cells examined (4 per cent) contained 59 markers. In Group III 77 of 750 cells (10 per cent) had marker-type aberrations. The relatively high percentage of cells with marker chromosomes in spermatogonial cells was expected because of the age of the rats at sacrifice (63 weeks). However, the values for Groups II and III clearly indicated a significant increase in the formation of markers. Furthermore, the increase was dose-related. There were significant increases in numbers of spermatogonial cells showing markers produced in both treated groups. The number of animals that had marker-type aberrations was high in the control group (9/35 or 26 per cent), but Group II had 20 of 35 animals (57 per cent) showing marker aberrations. Of the 30 animals evaluated in Group III, 25 (83 per cent) showed marker aberrations. The effect was clearly widespread in both low- and high-anesthetic-concentration groups, and was not limited to only a few animals.

Approximately 100 metaphases were scanned on each bone marrow slide for an estimate of polyploidy. The control group included only one animal with two cells that showed polyploidy. Group II included two animals with one cell each that was polyploid. There was no evidence of any polyploidic cell in Group III. Other incidental findings included sporadic nonclonal hypodiploidy, which was observed at comparable frequencies among all three groups. An estimate of polyploidy was made for every analyzed spermatogonial cell slide in all groups. Polyploidy was observed at comparable frequencies among the three groups. In addition to polyploidy, other observations included sporadic nonclonal hypodiploidy, which was also observed in equal frequencies among the groups.

Figure 1 is a representative photomicrograph of a metaphase from a spermatogonial cell from Group I. Figure 2 is a similar representative metaphase from Group III. The difference between the normal discrete, ordered chromosomal array and the disordered, nondiscrete array is apparent. The arrows indicate classifiable chromosomal abnormalities.

Discussion

The findings in this study demonstrate, in an experimental model, that chronic exposure to trace levels of halothane combined with nitrous oxide in a proportion similar to that used in surgical anesthesia can have adverse effects on the reproductive process. Perhaps more importantly, the study demonstrated an effect on male chromosomes *in vivo*. Exposure of female rats to the combination resulted in decreased ovulation and implantation efficiency at the lower level of exposure (halothane, 1.0 ppm, plus N_2O, 50 ppm) as well as at the higher level. A slight developmental retardation was seen in the fetuses of the pregnant dams exposed from Day 6 to Day 15 of gestation. However, no major teratologic effect, as reported by Knill-Jones *et al.*[5] and by Cohen *et al.*[4] was observed. Indications of exposure-related post-implantation loss were less clear: a significant loss was seen in the higher-level group exposed from Day 1 through Day 15 of gestation, but the loss observed in those mated females exposed only from Day 6 to Day 15 was not statistically significant. Thus, while there was no compelling evidence of an abortifacient effect from exposure during organogenesis (or from 12 weeks of exposure prior to mating), there was a disruptive effect on pregnancy from exposure prior to the period of major organogenesis. This effect on the gestation index would appear to have been determined by the exposure of the female; no dominant-lethal mutagenic or other abortifacient effect from the 12 weeks of exposure of the males prior to mating was evident. This latter negative finding is in line with the report by Wharton *et al.*[10] that the reproductive performance of the male mouse was unaffected by 17 weeks of exposure to halothane, 3,000 ppm, for four hours/day. Our negative finding might seem surprising in view of the significant chromosomal effects seen in spermatogonial cells in the present study. However, it must be

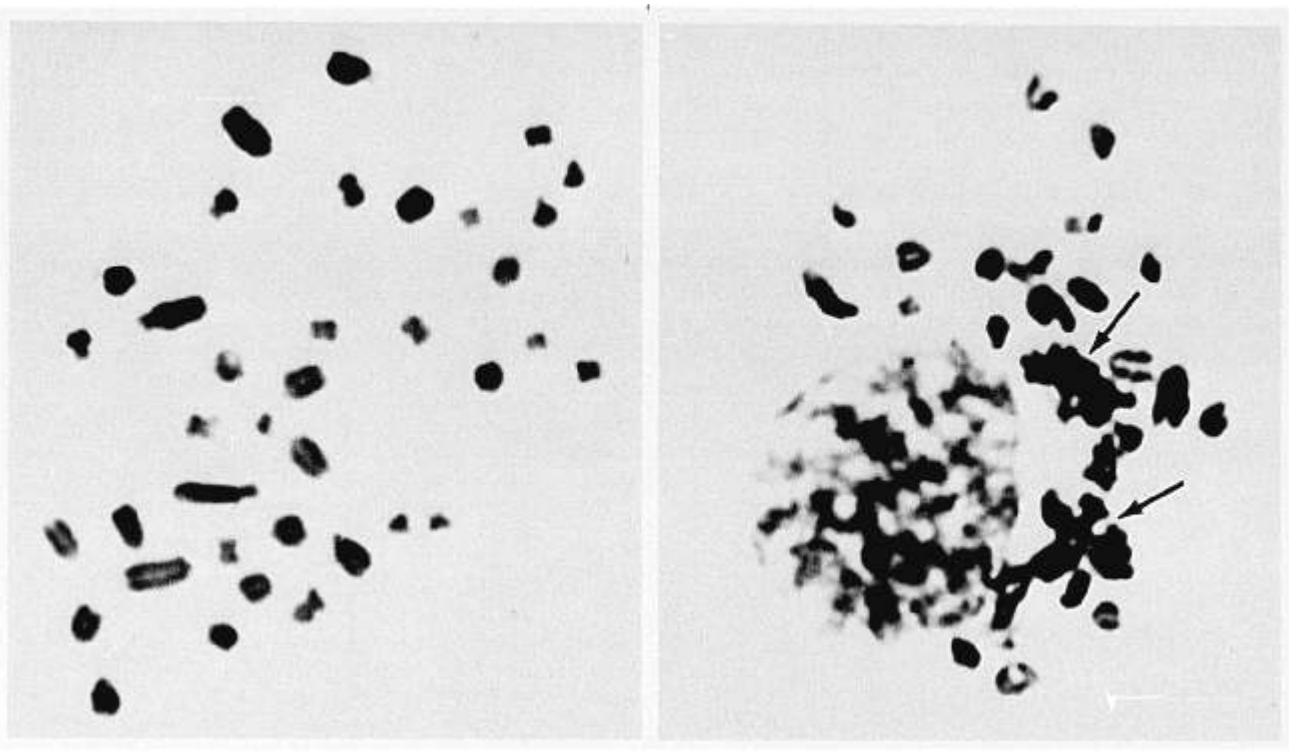

FIG. 1 (*left*). Typical normal spermatogonial metaphase from a Group I rat.

FIG. 2 (*right*). Spermatogonial metaphase from a Group III rat, showing two large exchange figures (*arrow*) and other chromosomal damage.

noted that the cytogenetic effects were seen only after a much longer period of exposure. Whether they would have been seen after 60 days of exposure is moot.

There was no effect on the durations of gestation of those females successfully carrying to term. This finding substantiated the unshortened gestational time reported for operating room personnel.[4]

A significant decrease in weight gain was seen at weaning in the male offspring of females exposed before and during pregnancy to the high-concentration combination of halothane and N_2O. It could not be ascertained whether this was a function of the female or male parental exposures or due to the greater survival rate in the exposed group, *i.e.*, its higher lactation index.

Significant increases in numbers of animals and in numbers of cells per animal with chromosomal aberrations (excluding gaps) were observed in both groups of male rats exposed to the halothane–N_2O mixture. This effect was seen in both bone marrow and spermatogonial cells. To our knowledge, this is the first report of chromosomal damage from these combined agents in an *in-vivo* test system. Since the slides were read blind and separately, the fact that the effect was seen in both cellular systems is noteworthy.

No attempt was made to mate the male rats after 52 weeks of exposure, so it was not possible to assess the genetic significance of the chromosomal damage observed in spermatogonial cells. A possible explanation for the failure of earlier exposure of the male rats to cause post-implantation loss is that, at the time of mating, they had been exposed for only 12 weeks (60 exposure days), whereas at the time the testes were prepared for cytogenetic analysis, they had been exposed for 52 weeks (≈260 exposure days), and that the longer exposure was necessary to allow cumulative interaction of the toxin(s) and aging cellular material. Clearly, it would be desirable to have a chronic exposure with adult male rats in which the mating and the cytogenetic phases were coordinated (*e.g.*, mating after 260 days of exposure followed by immediate sacrifice), even though this might be expected to decrease spermatogenesis or potency due to their more advanced age.

The cytogenetic effects obtained in both bone marrow and spermatogonial cells raises the question of a possible carcinogenic effect of chronic exposure to low concentrations of halothane plus N_2O. This question was the subject of a companion study in which male and female rats were exposed (without tumorigenic effect) for 24 months (520 days) to the same concentrations of these anesthetic gases.

Even in the absence of a tumorigenic effect in this

species, the fact that combined levels of halothane and N_2O as low as 1 ppm and 50 ppm, respectively, could produce significant chromosomal damage in bone marrow and spermatogonial cells emphasizes the desirability of a standard for occupational exposure to combined halothane and N_2O.*** This study did not address the question of whether N_2O or halothane alone could cause the effects observed. That the results of this experimental study did not confirm many of the reported epidemiologic findings should not be taken to negate the latter. Rather, species differences, as well as possible concomitant influences, such as stress, in the working lives of anesthesiologists and anesthetists[17] may be responsible for the discrepancies.

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*** A standard of halothane, 0.2 ppm, plus N_2O, 25 ppm, has been recommended by the National Institute for Occupational Safety and Health.