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Fetal Morphology in Mice Exposed to Halothane

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The teratogenic potential of subanesthetic and anesthetic exposure to halothane was studied in Swiss/ICR mice. Two treatment regimens were employed: daily exposure of males and females for nine weeks prior to conception and on days 1 through 17 of pregnancy; and exposure of females only on days 6 through 15 of pregnancy. Mice were exposed to subanesthetic concentrations of halothane for 0.025, 0.1, 0.4, and 1.2 MAC hours/day; anesthetic exposure was 4.0 MAC hours/day. Fetal morphologic development was normal at the two lowest exposures. Exposures of 0.4 MAC hours/day and more were associated with decreased fetal ossification. At the 1.2 MAC hour/day exposure, renal pelvic maturation was retarded and the incidence of skeletal variants was increased. The incidences of major malformations and minor anomalies were not increased following exposure to subanesthetic concentrations of halothane. Anesthetic exposure to 4.0 MAC hours/day was lethal to both dams and embryos, and resulted in major developmental malformations in surviving fetuses. These effects were probably due to altered maternal physiologic status. It is concluded that exposure of mice to subanesthetic concentrations of halothane does not result in important morphologic abnormalities in their offspring. (Key words: Anesthetics, volatile: halothane. Toxicity: fetal; teratogenicity; trace concentrations.)

THE RATES OF INFERTILITY, spontaneous abortions, stillbirths, and congenital malformations of offspring are increased in operating room workers.[1–9] Exposure to trace concentrations of anesthetic agents has been suggested, but not established, as the cause of these abnormalities. Numerous animal studies have examined the reproductive effects of anesthetic agents,[10–19] but no study has adequately examined the teratogenicity of halothane at subanesthetic and anesthetic levels. In a previous study we examined the overall effects of exposure to halothane on reproduction.[19] In the present study we have investigated the teratogenic potential of halothane.

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Materials and Methods

Virgin Swiss/ICR mice¶ were marked with metal ear tags, quarantined for seven days, then randomly assigned to experimental groups. Mice were housed four per cage by sex and treatment group, and bedded on ground corn cob.** Room temperature was maintained at 21 ± 1 C and artificial lighting was provided from 6 AM to 7 PM each day. No other animal species or mouse strains were housed in the same room, and no pesticides or germicides were used. Food†† and water were available at all times except during treatment periods. Mice were weighed weekly. Food and water intake were not measured because of the large numbers of mice involved.

Inhalational exposures were performed in two gas-tight stainless steel and plexiglass chambers, each of 1,500-l capacity; two exposures were begun daily at 7 AM and two at 12 noon. All mice in a treatment group were exposed simultaneously. The floor of each chamber was covered with soda lime to absorb carbon dioxide. Cages were placed randomly in the chambers. Halothane was vaporized in a bubble-through vaporizer with medical-grade compressed air delivered at a flow of 3–6 l/min through rubber tubing. Uniform anesthetic vapor concentration was maintained in each chamber by a high-volume recirculation fan. Halothane concentrations were monitored at 5- to 15-min intervals using a Varian 1440 gas chromatograph, and were maintained within 10 per cent of the desired concentrations.

Three sets of experiments were performed. Experiment A consisted of exposure of mice to low subanesthetic concentrations of halothane from prior to conception to completion of gestation. Five-week-old male and female mice were randomly divided into three control and three treatment groups (table 1, Groups 1–6). Colony controls, Group 1, were left undisturbed in the animal room and were untreated; treatment control females, Group 2, were exposed to compressed air for four hours daily in an inhalation chamber; positive control females, Group 3, were treated on day eight of pregnancy by gavage with

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TABLE 1. Exposure Schedule for Teratology Studies

	Agent	Hours/ Day	MAC Hr/Day	Weeks of Exposure Prior to Mating	Gestation Days Exposed
Experiment A					
Group 1	No treatment (colony control)	—	—	—	—
Group 2	Compressed air (treatment control)	4	—	9	1 through 17
Group 3	Retinoic acid (positive control)	—	—	—	8
Group 4	Halothane, 0.05 per cent	0.5	0.025	9	1 through 17
Group 5	Halothane, 0.05 per cent	2	0.1	9	1 through 17
Group 6	Halothane, 0.1 per cent	4	0.4	9	1 through 17
Experiment B					
Group 7	No treatment (colony control)	—	—	—	—
Group 8	Compressed air (treatment control)	4	—	9	1 through 17
Group 9	Halothane, 0.1 per cent	4	0.4	9	1 through 17
Group 10	Halothane, 0.3 per cent	4	1.2	9	1 through 17
Group 11	Halothane, 1.0 per cent	4	4.0	9	1 through 17
Experiment C					
Group 12	No treatment (colony control)	—	—	—	—
Group 13	Compressed air (treatment control)	4	—	—	6 through 15
Group 14	Retinoic acid (positive control)	—	—	—	8
Group 15	Halothane, 0.1 per cent	4	0.4	—	6 through 15
Group 16	Halothane, 0.3 per cent	4	1.2	—	6 through 15
Group 17	Halothane, 1.0 per cent	4	4.0	—	6 through 15

retinoic acid, a known teratogen, in corn oil, 15 mg/kg. The latter group was included to demonstrate the susceptibility of the mouse strain to induced malformations. Mice in Groups 4, 5, and 6 were exposed to halothane, 0.05 per cent for half an hour daily (0.025 MAC hour/day), 0.05 per cent for two hours daily (0.1 MAC hour/day), and 0.1 per cent for four hours daily (0.4 MAC hour/day), respectively. Following the ninth week of treatment, females were recaged in pairs and each pair was mated nightly for seven nights with one male from the same treatment group. Each morning females were examined for vaginal copulatory plugs. The day a copulatory plug was observed was considered day "0" of pregnancy. Females without plugs were mated for an additional seven nights with a different male. Daily inhalational exposures of females were continued through day 17 of pregnancy.

Experiment B consisted of exposure of mice to high subanesthetic and anesthetic concentrations of halothane from prior to conception to completion of gestation. Five-week-old mice were randomly divided into five groups (table 1, Groups 7–11). Groups 7 and 8 were colony and treatment control groups, respectively. Mice in Groups 9, 10, and 11 were exposed to halothane, 0.1 per cent for four hours daily (0.4 MAC hour/day), 0.3 per cent for four hours daily (1.2 MAC hours/day), and 1.0 per cent for four hours daily (4.0 MAC hours/day), respectively. All exposures were administered five days/week for nine weeks prior to conception and daily throughout day 17 of pregnancy. Matings were performed as in Experiment A.

Experiment C consisted of exposure of mice to high subanesthetic and anesthetic concentrations of halothane during organogenesis only. Ten-week-old previously untreated virgin mice were mated nightly, two females to one male, until copulation occurred or for a maximum of seven nights. Pregnant mice were randomly assigned to one of six groups (table 1, Groups 12–17). Levels of halothane exposure were the same as in Experiment B. However, in this experiment, dams were exposed only on days 6 through 15 of pregnancy, the period of fetal organogenesis. A positive control group was included as in Experiment A.

In all three experimental groups, each dam was killed by cervical dislocation on day 18 of pregnancy. The uterus was examined and the numbers and positions of live and dead fetuses and resorptions were recorded. Crown–rump length, weight, and sex of each live fetus was determined, and each fetus was examined for external abnormalities. One third (Experiment A) or two thirds (Experiments B and C) of the live fetuses were randomly selected, cleared with potassium hydroxide, and stained with alizarin red S using the method of Staples and Schnell,[20] and subsequently examined for skeletal anomalities. The remainder of the fetuses were preserved in Bouin's fixative solution and subsequently dissected and examined for internal soft-tissue abnormalities as described by Barrow and Taylor.[21] In all cases, fetuses

TABLE 2. Classification of Fetal Abnormalities

External Examination	Skeletal Examination	Internal Examination
Runt	Major malformations	Major malformations
Major malformations	Craniofacial	Minor anomalies
Cleft palate	Ribs or vertebrae	Enlarged brain ventricle
Exencephaly	Other	Hemorrhage
Limb deformity	Minor anomalies	Gonad displacement
Other	Ribs or vertebrae	Bladder distention
Minor anomalies	Sternebrae	Other
Hematoma	Other	Developmental variant
Limb malposition	Developmental variant	Increased renal pelvic cavitation
Crooked tail	Supernumerary rib	Other
Other	Asymmetric or bipartite sternum	
	Other	
	Decreased ossification	
	Skull	
	Limbs	
	Sternum	
	Other	

were examined by an observer without knowledge of the treatment groups.

Abnormalities were classified by type and severity (table 2). Fetuses weighing 25 per cent less than the litter mean were classified as runts. Fetal morphologic abnormalities that would have precluded normal survival were considered major malformations, while abnormalities that were neither severely disfiguring nor incapacitating were classified as minor anomalies. The term developmental variant was used to describe distinct variations in normal development that are common in untreated animals, but could occur with increased frequency as a consequence of exposure to teratogens, *e.g.*, supernumerary ribs. Decreased ossification was classified separately from other skeletal variants since we wished to distinguish a process that only delayed fetal maturation from one that altered fetal morphology.

The percentage of fetuses affected in each litter was computed for each type of abnormality. Intergroup comparisons were made employing the Mann-Whitney *U* test; the litter was used as the basic experimental unit, and the proportion of abnormal fetuses per litter was the variable for analysis. The colony control and treatment control groups were compared with each other, and each halothane-treatment group was compared with the treatment control group for that experiment. The level of statistical significance used was 1 per cent, since the possibility of one or more false-positive results is great when multiple comparisons are performed at the usual 5 per cent level.

Three control and five halothane-exposed dams delivered prior to cesarean section and cannibalized one or more pups; data from these litters were omitted from analysis. Three control and two halothane-exposed dams were killed prematurely, and their litters were likewise excluded.

Results

In Experiments A and B, daily treatment with compressed air (treatment control) did not increase the incidence of abnormal offspring compared with the colony control group (table 3). In Experiment B, the incidence of decreased ossification was greater in the colony control group than in the treatment control group. Since this finding occurred in only one of three experiments, it was considered to be a random occurrence. The incidence of runts, major malformations, and minor anomalies were not increased at subanesthetic levels of halothane. Fetuses from dams exposed to 0.4 MAC hours/day (Groups 6 and 9) and 1.2 MAC hours/day (Group 10) showed dose-related increases in the incidence of decreased ossification. In addition, Group 10 fetuses had increased incidences of delayed renal pelvic maturation (increased renal pelvic cavitation), and skeletal variants, including supernumerary ribs and sternal ossification defects. Only one mouse became pregnant following daily four-hour exposures to halothane, 1.0 per cent (Group 11). All four of the live pups from this dam had severe growth retardation. Treatment with retinoic acid (Group 3) resulted in increased major malformations, including cleft palate and exencephaly.

In Experiment C, there was no difference in the incidences of fetal abnormalities between the colony control group and the treatment control group exposed to compressed air on days 6 through 15 of pregnancy (table 4). The overall incidence of fetuses with skeletal abnormalities was increased following exposure to 0.4 MAC hours/day (Group 15); however,

TABLE 3. Experiments A and B, Fetal Abnormalities Following Exposure to Halothane from Prior to Conception to Completion of Gestation

Exposure	Experiment A						Experiment B				
	Group 1 Colony Control	Group 2 Treatment Control	Group 3 Positive Control	Group 4 MAC Hours per Day 0.025	Group 5 MAC Hours per Day 0.1	Group 6 MAC Hours per Day 0.4	Group 7 Colony Control	Group 8 Treatment Control	Group 9 MAC Hours per Day 0.4	Group 10 MAC Hours per Day 1.2	Group 11† MAC Hours per Day 4.0
External examination											
Number of fetuses (litters)	313(28)	291(28)	80(9)	252(25)	339(30)	314(29)	326(29)	307(28)	314(29)	173(19)	4(1)
Any external abnormality (per cent)*	6.3	4.0	24.0‡	2.8	1.3	4.2	2.3	1.3	3.9	7.3	100.0
Runt	0.9	1.6	0.0	0.6	0.8	2.0	0.8	0.4	1.5	1.8	0.0
Major malformation	0.4	0.3	23.0‡	0.6	0.2	0.3	0.0	0.3	0.6	1.6	0.0
Minor anomaly	4.9	2.5	2.0	1.6	0.5	2.2	1.5	0.6	2.1	4.0	100.0
Skeletal examination											
Number of fetuses	105	102	26	87	113	105	217	205	205	116	3
Any skeletal abnormality (per cent)*	32.0	40.6	49.8	27.2	34.3	44.8	45.3	30.8	57.5‡	89.6‡	100.0
Major malformation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Minor anomaly	0.0	3.0	0.0	1.4	0.8	4.4	1.0	0.5	5.0	7.1	0.0
Developmental variant	29.3	35.6	49.8	27.2	28.8	40.4	31.0	28.4	42.8	59.0‡	66.7
Decreased ossification	2.7	2.1	0.0	0.0	7.5	11.0	17.5‡	2.4	25.1‡	68.8‡	100.0
Internal examination											
Number of fetuses	208	189	54	165	226	209	109	102	109	57	1
Any internal abnormality (per cent)*	4.5	2.4	4.6	2.5	1.8	3.8	10.1	4.8	5.5	27.6‡	100.0
Major malformation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Minor anomaly	1.3	0.4	0.0	1.5	0.4	0.0	3.1	1.2	0.9	0.0	0.0
Developmental variant	3.2	2.4	4.6	1.0	1.5	3.8	7.9	3.6	4.6	27.6‡	100.0

* Mean per cent of abnormal fetuses per litter.
† Group too small for statistical comparison.
‡ $P < 0.01$ *vs.* treatment control.

TABLE 4. Experiment C, Fetal Abnormalities Following Exposure to Halothane during Organogenesis

	Experiment C					
	Group 12 Colony Control	Group 13 Treatment Control	Group 14 Positive Control	Group 15 MAC Hours per Day 0.4	Group 16 MAC Hours per Day 1.2	Group 17† MAC Hours per Day 4.0
External examination						
Number of fetuses (litters)	295(27)	284(27)	98(9)	245(24)	214(19)	5(1)
Any external abnormality (per cent)*	3.6	6.9	49.1‡	9.6	9.2	100.0
Runt	0.0	0.0	0.0	0.3	0.0	0.0
Major malformation	0.7	0.6	43.7‡	0.0	1.2	100.0
Minor anomaly	2.9	6.3	2.0	9.3	7.7	0.0
Skeletal examination						
Number of fetuses	198	191	65	158	145	3
Any skeletal abnormality (per cent)*	36.3	29.2	92.6‡	50.9‡	45.8	100.0
Major malformation	0.0	0.0	2.8	0.0	0.0	33.3
Minor anomaly	0.4	0.5	28.1‡	2.8	1.8	66.7
Developmental variant	35.5	27.9	90.7‡	48.7	41.4	100.0
Decreased ossification	0.5	0.9	0.0	1.3	3.5	100.0
Internal examination						
Number of fetuses	97	93	33	87	69	2
Any internal abnormality (per cent)*	12.8	12.0	14.8	10.9	14.2	100.0
Major malformation	0.0	0.0	0.0	0.0	0.0	50.0
Minor anomaly	5.7	5.3	5.6	5.4	7.2	100.0
Developmental variant	7.1	6.8	9.3	9.8	7.0	0.0

* Mean per cent of abnormal fetuses per litter.
† Group too small for statistical comparisons.
‡ $P < 0.01$ *vs.* treatment control.

no specific classification of skeletal abnormality was significantly increased. No effect was observed after 1.2 MAC hours/day exposure (Group 16). Daily anesthetic exposure to 4.0 MAC hours/day (Group 17) was lethal to 14 of 23 pregnant females. One hundred per cent embryolethality resulted in eight of the nine surviving dams. All five fetuses from the ninth dam were malformed. There were two fetuses with cleft palate, three with anophthalmia, two with cranial asymmetry, and two with limb deformities. All three fetuses subjected to skeletal examination had multiple fused vertebrae or fused ribs. The offspring of retinoic acid-treated dams (Group 14) showed increased incidences of major external malformations, minor skeletal anomalities, and skeletal variants.

Discussion

Offspring of animals exposed to as much as 0.1 MAC hours/day of halothane prior to conception and throughout pregnancy showed no evidence of altered morphology. Exposures to 0.4 MAC hours/day or more resulted in dose-related retardation of fetal maturation; exposure to 1.2 MAC hours/day resulted in an increased incidence of skeletal developmental variants. When dams were exposed only during the period of organogenesis (Experiment C), retardation of fetal maturation did not occur, nor was the incidence of skeletal variants increased. None of the effects observed following exposure to subanesthetic concentrations of halothane would be expected to result in permanent abnormality or in decreased survival. Thus, in mice, there is no teratogenic hazard from halothane exposure at levels comparable to those found in unscavenged operating rooms.

In contrast to the paucity of effects at subanesthetic levels, there was marked embryotoxicity associated with exposure to anesthetic concentrations of halothane. The anesthetized mice became markedly hypothermic, with core temperatures as low as 24 C following treatment, and were presumed to have significant metabolic, circulatory, and respiratory alterations. Maternal nutritional status may also have been impaired by the repeated anesthetizations. Fifty-eight per cent of all pregnant mice exposed to anesthetic concentrations of halothane died before day 18 of pregnancy. The associated embyrotoxicity in survivors was probably the result of altered maternal physiologic status or nutritional impairment rather than a direct effect of halothane on the developing embryo. It is likely that all inhalational anesthetics, when administered daily at anesthetic concentrations, will produce similar embryotoxic effects. Effects produced at anesthetic concentrations, however, must be differentiated from those that result from subanesthetic exposure.

Three earlier studies examined the reproductive toxicity of trace or subanesthetic concentrations of halothane in rodents. Bruce[14] demonstrated no effect on fertility or reproduction when mice were exposed to halothane, 0.0016 per cent, for seven hours daily (0.011 MAC hour/day), five days per week, for six weeks prior to mating and during pregnancy. Lansdown *et al.*[17] demonstrated no fetotoxicity or increased frequency of skeletal anomalies in offspring of Sprague-Dawley rats exposed to halothane concentrations as high as 0.32 per cent for eight hours per day on days 8 through 12 of pregnancy. Exposure of dams to halothane, 0.16 per cent, on days one through 21 of pregnancy resulted in retarded fetal growth, but there was no increase in skeletal anomalies. In the third study, Wharton *et al.*[19] examined fertility, reproductive performance and postnatal survival in Swiss/ICR mice exposed as in Experiments A and B of the present study. Dose-related decreases in maternal weight gain and fetal weight and length resulted from halothane exposures of 0.4 MAC hours/day or more. Pregnancy rate, implantation rate, and number of live fetuses per litter were significantly decreased at 1.2 MAC hours/day. The percentage of resorptions or fetuses dead *in utero* was not increased; postnatal survival of offspring was not affected.

Other investigators have studied the reproductive effects of exposure to halothane at anesthetic concentrations. Basford and Fink[12] reported an increased incidence of skeletal variants, *i.e.*, lumbar ribs and vertebral anomalities, in rats exposed to halothane, 0.8 per cent, in oxygen, 25 per cent, for 12 hours on days 8, 9.5, or 10 of pregnancy. Bussard *et al.*[15] demonstrated decreased mean fetal length and weight in hamsters exposed to nitrous oxide, 60 per cent, plus halothane, 0.6 per cent, for three hours on day 10 or 11 of pregnancy. Increased embryolethality was seen in hamsters exposed on day 11. Fetal morphology was not examined. Kennedy *et al.*,[16] however, demonstrated no adverse effect on reproduction in rats following one-hour exposures to halothane, 1.4 per cent, for five consecutive days, either prior to mating or during early, mid- or late pregnancy. Additionally, no adverse effect was demonstrated in rabbits exposed to halothane, 2.2 per cent, for one hour on four or five consecutive days during early or mid-pregnancy.

The greater incidence of embryotoxicity at anesthetic concentrations demonstrated in the present study is undoubtedly due to differences in experimental design with respect to: species tested; concentration, duration and frequency of anesthetic exposures; timing of exposures relative to the period of fetal development; number and types of fetal morphologic observations. The present study is in accordance with U. S. Food and Drug Administration guidelines for

studies of reproduction.[22] Exposure schedules were designed to detect adverse effects of maternal or paternal exposure during any phase of the reproductive process. Multiple levels of exposure were studied to define a dose–response relationship for any observed effect, while large treatment groups were used to increase the probability that an effect, if present, would be detected. Finally, a positive control group was included to demonstrate the susceptibility of the mouse strain to induced malformations.

We conclude that there is no experimental evidence to indicate that exposure to subanesthetic concentrations of halothane is significantly embryotoxic or teratogenic. Specifically, neither fetal lethality nor the frequency of major or minor congenital malformations was increased in our studies. Moreover, there is a wide margin of safety between the levels of halothane that are present in unscavenged operating rooms and those that result in detectable alterations in rodent embryonic development.

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