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Reproduction and Fetal Development in Mice Chronically Exposed to Enflurane

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Reproductive indices and developmental toxicity were evaluated in Swiss/ICR mice chronically exposed to a subanesthetic (0.01 or 0.1 per cent) or an anesthetic (0.5/1.0 per cent) concentration of enflurane. Pregnant mice (443) and fetuses (4743) were examined. In one experiment, groups of females were exposed to 0.01, 0.1, or 0.5/1.0 per cent enflurane for 4 hours per day, seven days per week for 3 weeks; they were then mated with unexposed males. Exposure of females was continued daily throughout pregnancy. No adverse effects on fertility were observed at any dosage. At the highest dosage, 1.0 per cent, minor developmental variations occurred (*i.e.*, lumbar ribs and increased renal pelvic cavitation). In a second experiment, groups of mice were exposed to 0.01, 0.1 or 1.0 per cent enflurane only on days 6 through 15 of pregnancy for 4 hours per day, after having been mated with untreated males. Abnormalities (*i.e.*, increased incidence of cleft palate, minor skeletal and visceral anomalies, and developmental variants) were again seen only at the highest dosage. In a third experiment, male mice were exposed to 0.01, 0.1, or 0.5/1.0 per cent enflurane for 11 weeks for 4 hours per day, 5 days per week, prior to mating with unexposed females; results of this experiment were negative. In general, enflurane treatments did not adversely affect reproductive indices. Effects on fetal development were minimal, being somewhat greater than those reported in previous experiments with methoxyflurane but less than those seen with halothane. The smallest exposure at which effects were seen was approximately 100 times greater than the level of human occupational exposure in unscavenged operating rooms. (Key words: Anesthetics, volatile: enflurane; trace concentrations. Toxicity: fetal; teratogenicity; reproductive; trace concentrations.)

RATES OF INFERTILITY, spontaneous abortion, and congenital abnormalities of offspring among operating room personnel have been reported to be increased.[1–5] Occupational exposure to trace concentrations of waste anesthetic gases has been suggested, but not established, as the cause of these increases. The reproductive toxicity of chronic exposure to trace, subanesthetic and anesthetic concentrations of halothane and methoxyflurane has previously been assessed in our laboratory[6–8] and those of others.[9–12] In the present study, we examine the effects of chronic enflurane exposure on reproduction and fetal development in Swiss/ICR mice.

Materials and Methods

Virgin Swiss/ICR mice§ were quarantined for seven days, then marked with metal ear tags and four males or four females were housed in each cage. Room temperature was maintained at 21 ± 1° C and artificial lighting was provided for thirteen hours each day. No other animal species or mouse strains were housed in the same room, and no germicides or pesticides were used. Mice were fed a standard laboratory animal diet¶ and tap water, *ad libitum*, except during treatment periods, and were bedded on ground corncob.** Food and water intake were not measured because of the large numbers of mice involved. All mice were weighed weekly; pregnant females were weighed on days 0, 7, 14 and 18 of pregnancy. Inhalational exposures were performed in two gas-tight stainless steel and Plexiglass® chambers, each with a capacity of 1,500 liters; two exposures were started daily at 8 A.M., and two at 1 P.M. All mice in a treatment group were exposed simultaneously. Cages were placed randomly in the chambers. Enflurane was vaporized in a bubble-through vaporizer with medical-grade compressed air and delivered at a flow of 6 l/min through rubber tubing. Uniform anesthetic vapor concentration was maintained in each chamber by a high-volume recirculation fan. Enflurane concentrations were monitored at 15-min intervals using a Varian® 1440 gas chromatograph, and were maintained within 10 per cent of the desired concentrations.

EXPERIMENT A: EXPOSURE OF FEMALE MICE FROM PRIOR TO CONCEPTION TO COMPLETION OF GESTATION

Females were randomly assigned to experimental groups at six weeks of age (table 1). Enflurane-treatment groups were exposed to enflurane, 0.01 per cent (100 ppm), 0.1 per cent (1,000 ppm), or 1.0 per cent (10,000 ppm) for 4 hours per day, 7 days per week for 3 weeks prior to mating. Control mice were either un-

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¶ Tab Litter®, Paxton Processing Co., Paxton Illinois 60957.
** Wayne Lab Blox, Allied Mills, Inc., Chicago, Illinois 60606.

TABLE 1. Exposure Schedule for Enflurane Reproduction Studies

Treatment	Hours/Day	MAC Hours/Day	Days/Week	Weeks of Exposure Prior to Mating: Males	Weeks of Exposure Prior to Mating: Females	Gestation Days Exposed
Experiment A						
No treatment (colony control)	—	—	—	—	—	—
Compressed air (treatment control)	4	—	7	—	3	0–17
Enflurane, 0.01 per cent	4	0.02	7	—	3	0–17
Enflurane, 0.1 per cent	4	0.2	7	—	3	0–17
Enflurane, 1.0/0.5 per cent*	4	2.0/1.0	7	—	3	0–17
Retinoic acid, 15 mg/kg (positive control)	—	—	—	—	—	8
Experiment B						
No treatment (colony control)	—	—	—	—	—	—
Compressed air (treatment control)	4	—	7	—	—	6–15
Enflurane, 0.01 per cent	4	0.02	7	—	—	6–15
Enflurane, 0.1 per cent	4	0.2	7	—	—	6–15
Enflurane, 1.0 per cent	4	2.0	7	—	—	6–15
Retinoic acid, 15 mg/kg (positive control)	—	—	—	—	—	8
Experiment C						
Compressed air (treatment control)	4	—	5	11	—	—
Enflurane, 0.01 per cent	4	0.02	5	11	—	—
Enflurane, 0.1 per cent	4	0.2	5	11	—	—
Enflurane, 1.0/0.5 per cent†	4	2.0/1.0	5	11	—	—

* 1.0 per cent prior to mating; 0.5 per cent throughout gestation.
† 1.0 per cent for 5.5 weeks; 0.5 per cent thereafter.

treated (colony control), treated with compressed air in an inhalation chamber (treatment control) or treated with retinoic acid, 15 mg/kg, in corn oil by gavage on day 8 of pregnancy (positive control). At age 9 weeks, females were recaged two per cage, and mice were mated nightly for 7 nights (one untreated male to two females). Females were examined each morning for presence of a copulatory plug, which signified day 0 of pregnancy. Females which had not copulated after 7 nights of mating were mated for 7 additional nights with a different male. Treatments continued throughout mating and gestation. The highest enflurane dose, 1.0 per cent, was reduced to 0.5 per cent at the beginning of mating because the females in this group were failing to gain weight.

On day 18, one day before the mice were due to deliver, each dam was weighed and then killed by cervical dislocation. 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 were determined, and each fetus was examined for external abnormalities. Two-thirds of the live fetuses were randomly selected to be cleared with potassium hydroxide, stained with alizarin red S using the method of Staples and Schnell,[13] and subsequently examined for skeletal abnormalities. 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.[14] All examinations were done without knowledge of the treatment groups. Fetal abnormalities were classified as in previous studies.[7,8]

EXPERIMENT B: EXPOSURE OF FEMALE MICE ON DAYS 6 THROUGH 15 OF GESTATION ONLY

Nine-week-old mice were mated nightly, 1 male to 2 females, until copulation occurred or for a maximum of 7 nights. On day 0 of pregnancy, each female was recaged, if necessary; so that each cage contained two females impregnated on the same day; each pair of pregnant mice was then randomly assigned to an experimental group. Groups were similar to those in Experiment A, except that mice were treated only on days 6 through 15 of gestation and the group treated with the highest concentration received 1.0 per cent throughout. Uterine and fetal examinations were done as in Experiment A.

EXPERIMENT C: EXPOSURE OF MALE MICE FOR ELEVEN WEEKS PRIOR TO MATING

Four-week-old males were randomly assigned to three enflurane groups, as in the other experiments, and a treatment control group. They were treated four hours per day, five days per week, for 11 weeks prior to mating. Males in the group receiving enflurane, 1.0 per cent, were slow to gain weight, so the level was reduced to 0.5 per cent after 5.5 weeks. Treatments continued while the males were mated

TABLE 2. Experiment A. Reproduction Indices Following Exposure of Female Mice to Enflurane from Prior to Conception to Completion of Gestation, Mean ± SE

	Colony Control	Treatment Control	Enflurane			Positive Control
			0.01 Per Cent	0.1 Per Cent	1.0/0.5* Per Cent	
Number of females	48	48	40	40	39	24
Premating weight gain (g)	1.8 ± 0.1	1.7 ± 0.3	1.3 ± 0.2	1.8 ± 0.2	−0.4 ± 0.2†	2.0 ± 0.2
Pregnancy rate (per cent)	90	92	93	100	95	96
Gestational weight gain (g)	27.6 ± 0.8	25.6 ± 0.6	26.9 ± 0.6	25.9 ± 0.6	24.0 ± 0.7	26.1 ± 1.0
Implantations/Dam	13.6 ± 0.5†	12.2 ± 0.4	13.2 ± 0.3	12.0 ± 0.3	12.0 ± 0.4	12.9 ± 0.4
Live fetuses/Litter	12.2 ± 0.4	11.2 ± 0.4	12.1 ± 0.3	10.9 ± 0.3	10.9 ± 0.4	10.7 ± 0.6
Reproductive loss						
Resorbed (per cent)	9.7 ± 1.3	7.7 ± 1.7	8.7 ± 1.4	8.3 ± 1.4	9.7 ± 1.9	17.2 ± 2.7†
Dead *in utero* (per cent)	0.2 ± 0.2	0.4 ± 0.3	0.0	1.0 ± 0.6	0.4 ± 0.4	0.4 ± 0.4
Mean fetal weight (g)	1.31 ± 0.01	1.34 ± 0.02	1.33 ± 0.02	1.33 ± 0.01	1.28 ± 0.02	1.30 ± 0.02

* 1.0 per cent prior to mating; 0.5 per cent throughout gestation. † $P < 0.05$ *vs.* Treatment Control.

to untreated virgin females. Uterine and external fetal examinations were done as in Experiment A.

The percentage of fetuses affected in each litter was computed for each type of abnormality. Intergroup comparisons were made employing analysis of variance and the Mann-Whitney U test, as appropriate. 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 enflurane-treated group was compared with the treatment control group for that experiment. $P < 0.05$ was considered significant.

Results

Experiment A: Exposure of Female Mice from Prior to Conception to Completion of Gestation

Mice exposed to 0.01 and 0.1 per cent enflurane showed no change in behavior during treatment. Enflurane, 1.0 per cent, induced ataxia followed by light sleep; however, mice were awake within 15 min after starting to exhaust the chamber. Enflurane, 0.5 per cent, caused the mice to remain hyperactive throughout the period of exposure. Pregnancy rates were the same in all groups (table 2). The mean number of implantations per dam was greater in the colony control than in the treatment control group (chance occurrence by historical data[6–8]); however, there were no differences between the enflurane-exposed and the treatment control groups. There were no differences among the five groups in the number of live fetuses per dam, percentage of resorptions or fetuses dead *in utero*, or mean fetal weight.

A dose-related increase in lumbar rib formation was seen, reaching statistical significance at the 1.0/0.5 per cent enflurane dose (table 3). Also, there was an increased incidence of delayed renal maturation (increased renal pelvic cavitation; IRPC). The positive control group had significantly higher percentages of fetal resorptions and fetuses with major external malformations (cleft palate and exencephaly), minor external abnormalities, minor skeletal abnormalities and developmental variants (mostly lumbar ribs).

Experiment B: Exposure of Female Mice on Days 6 through 15 of Gestation Only

There was no significant difference between the treatment control and the colony control groups in any of the variables examined (table 4). Enflurane exposure had no effect on implantation rate, number of live fetuses/dam, percentage of resorptions or fetuses dead *in utero*. Maternal gestational weight gain, mean fetal weight, and mean fetal length were decreased in the group exposed to enflurane, 1.0 per cent. Treatment with enflurane, 0.01 and 0.1 per cent, had no effect on fetal morphology (table 5). Treatment with enflurane, 1.0 per cent, resulted in increased incidences of major malformations (mostly cleft palate), minor skeletal anomalies (bent and fused ribs; fused vertebrae), skeletal variants (mostly lumbar ribs), decreased ossification, and visceral variants (mostly IRPC). Minor visceral anomalies also were significantly increased.

The positive control group had significantly higher percentages of fetal resorptions and fetuses with major external malformations (exencephaly and cleft palate), major and minor skeletal abnormalities, and skeletal variants.

Experiment C: Exposure of Male Mice for Eleven Weeks Prior to Mating

Results of this experiment were negative (table 6).

TABLE 3. Experiment A. Fetal Abnormalities Following Exposure of Female Mice to Enflurane from Prior to Conception to Completion of Gestation

	Colony Control	Treatment Control	Enflurane			Positive Control
			0.01 Per Cent	0.1 Per Cent	1.0/0.5* Per Cent	
Number of litters	37	37	33	37	37	20
External Examination						
Number of fetuses	452	415	398	402	403	214
Any external abnormality‡	2.9	3.0	2.5	4.1	4.2	55.0†
Runt	0.5	0.3	0.5	0.8	0.7	0.0
Major malformation	0.7	0.0	0.0	0.2	0.7	32.2†
Minor anomaly	1.8	2.7	2.1	2.8	2.8	39.5†
Skeletal Examination						
Number of fetuses	300	277	263	264	263	145
Any skeletal abnormality‡	25.9	31.4	27.7	38.1	44.7†	98.7†
Major malformation	0.3	0.0	0.0	0.0	0.4	0.0
Minor anomaly	0.9	1.1	0.7	2.3	1.0	86.2†
Developmental variant	24.1	26.8	24.6	33.4	41.0†	95.9†
Lumbar rib	17.4	20.5	15.6	24.0	33.7†	95.3†
Decreased ossification	3.5	6.7	4.3	6.1	8.3	7.2
Internal Examination						
Number of fetuses	151	137	132	138	137	67
Any internal abnormality‡	34.9	24.0	25.3	21.6	38.5†	22.5
Major malfunction	0.0	0.0	0.0	0.0	0.0	0.0
Minor anomaly	9.7	6.9	5.4	5.1	9.5	5.0
Developmental variant/IRPC	28.1	18.0	20.7	19.1	33.3†	20.0

* 1.0 per cent prior to mating; 0.5 per cent throughout gestation.
‡ Mean per cent abnormal fetuses per litter.
† $P < 0.05$ *vs.* Treatment Control.

Discussion

In the present study, exposure of Swiss/ICR mice to trace and subanesthetic enflurane concentrations did not adversely affect reproductive indices and fetal development. Only enflurane exposure which produced light general anesthesia, 1 per cent, was associated with fetotoxic effects. At that, these effects generally were minor except for the development of cleft palate in Experiment B mice. These results differ from those of a previous study of halothane[6,7] in which daily four-hour exposure to a subanesthetic concentration, 0.3 per cent, resulted in decreases in pregnancy rate, implantation rate and number of live fetuses per litter, and an increased incidence of skeletal developmental variants. The results more nearly agree with those of our study of the developmental toxicity of methoxyflurane in which only minor toxic maternal reproductive and fetal developmental effects were seen after exposure to a relatively greater anesthetic concentration, 0.2 per cent.[8] Although the three studies were not identically designed, they were similar enough to permit us to compare them and to allow us to suggest that the toxic thresholds for these

TABLE 4. Experiment B. Reproduction Indices Following Exposure of Female Mice to Enflurane on Days 6 through 15 of Gestation, Mean ± SE

	Colony Control	Treatment Control	Enflurane			Positive Control
			0.01 Per Cent	0.1 Per Cent	1.0 Per Cent	
Number of females	30	34	26	30	26	11
Pregnancy rate (per cent)	93	97	81	97	96	82
Gestational weight gain (g)	26.9 ± 0.8	26.1 ± 1.0	25.4 ± 1.2	26.6 ± 0.6	20.3 ± 0.5*	23.8 ± 1.7
Implantations/Dam	12.2 ± 0.3	11.9 ± 0.5	11.3 ± 0.6	12.7 ± 0.3	13.1 ± 0.3	12.9 ± 0.6
Live fetuses/Litter	10.9 ± 0.4	10.5 ± 0.5	10.0 ± 0.7	11.2 ± 0.3	11.5 ± 0.4	10.0 ± 0.5
Reproductive loss:						
Resorbed (per cent)	10.3 ± 1.6	12.3 ± 2.3	13.3 ± 3.3	11.3 ± 1.6	11.2 ± 1.9	21.5 ± 2.9*
Dead *in utero* (per cent)	0.0	0.3 ± 0.3	0.0	0.3 ± 0.3	0.9 ± 0.6	0.7 ± 0.7
Mean fetal weight (g)	1.32 ± 0.02	1.32 ± 0.02	1.36 ± 0.01	1.34 ± 0.01	1.03 ± 0.02*	1.30 ± 0.03
Mean fetal length (mm)	22.4 ± 0.3	23.1 ± 0.2	23.0 ± 0.3	22.7 ± 0.2	20.8 ± 0.3*	23.1 ± 0.5

* $P < 0.05$ *vs.* Treatment Control.

TABLE 5. Experiment B. Fetal Abnormalities Following Exposure of Female Mice to Enflurane on Days 6 through 15 of Gestation

	Colony Control	Treatment Control	Enflurane			Positive Control
			0.01 Per Cent	0.1 Per Cent	1.0 Per Cent	
Number of Litters	27	32	21	29	25	9
External Examination						
Number of fetuses	295	335	209	324	288	90
Any external abnormality*	4.2	3.4	1.0	4.2	7.2†	26.1†
Runt	0.3	1.0	0.0	0.8	0.9	1.1
Major malformation	0.0	0.0	0.6	0.6	2.6†	17.3†
Cleft palate	0.0	0.0	0.0	0.3	1.9†	12.6†
Minor anomaly	3.9	2.6	0.4	2.6	4.3	12.4†
Skeletal Examination						
Number of fetuses	195	220	138	217	194	58
Any skeletal abnormality*	28.2	30.5	22.3	28.1	75.5†	96.8†
Major malformation	0.0	0.0	0.0	0.0	0.0	1.6†
Minor anomaly	1.7	3.7	2.6	2.9	8.7†	19.7†
Developmental variant	25.0	26.2	19.7	25.2	53.5†	95.2†
Lumbar rib	16.4	19.5	6.1	17.3	38.6†	93.6†
Decreased ossification	1.0	2.4	0.0	1.3	53.5†	5.0
Internal Examination						
Number of fetuses	99	114	70	106	94	31
Any internal abnormality*	33.4	41.1	26.2	27.6	72.6†	53.7
Major malformation	0.0	0.0	0.0	0.0	1.0	0.0
Minor anomaly	18.1	17.4	10.6	13.6	26.9	36.1
Enlarged brain ventricle	5.6	7.6	2.5	2.0	21.5†	0.0
Developmental variant/IRPC	17.1	30.2	14.8	16.0	67.1†	17.6

* Mean per cent abnormal fetuses per litter.

† $P < 0.05$ *vs.* Treatment Control.

agents differ, methoxyflurane being the least toxic and halothane the most. It should be emphasized, however, that with all three agents there is a large margin of safety between the levels which are associated with even minor maternal or fetal abnormalities in mice and those which workers are exposed to in unscavenged operating rooms. For example, exposure of mice to 0.1 per cent enflurane for four hours (4,000 ppm · hr), a dose not associated with any abnormalities, results in an approximately 100-fold margin of safety when compared with a hypothetical situation of heavy operating room contamination, *i.e.*, 5–7 ppm for 6–8 hours (~40 ppm · hr). Given that in the present study, enflurane levels ten times higher (40,000 ppm · hr) were required before a significant number of abnormalities resulted, and that the average enflurane level in effectively scavenged operating rooms probably would be no more than 1 ppm, there is reason to believe that the drug poses little, if any, reproductive hazard to operating room workers. This statement, of course, must be considered in light of possible species differences in reproductive system thresholds of toxicity. Regarding that question, the present study draws some support

TABLE 6. Experiment C. Reproduction Indices Following Exposure of Male Mice to Enflurane for Eleven Weeks Prior to Mating, Mean ± SE

	Treatment Control	Enflurane		
		0.01 Per Cent	0.1 Per Cent	1.0/0.5* Per Cent
Number of males/Number of females	10/20	10/20	10/20	10/20
Males fertile (per cent)	100	100	100	100
Pregnancy rate (per cent)	90	100	85	95
Gestational weight gain (g)	27.5 ± 0.9	27.7 ± 0.8	29.4 ± 1.6	25.2 ± 1.3
Implantations/Dam	12.6 ± 0.4	13.1 ± 0.4	13.0 ± 0.7	11.4 ± 0.9
Live fetuses/Litter	11.3 ± 0.4	11.7 ± 0.4	11.6 ± 0.7	9.7 ± 0.9
Reproductive loss:				
Resorbed (per cent)	10.1 ± 2.4	10.4 ± 2.6	10.6 ± 1.9	13.5 ± 4.4
Dead *in utero* (per cent)	0.4 ± 0.4	0.0	0.5 ± 0.5	0.0
Mean fetal weight (g)	1.38 ± 0.03	1.37 ± 0.03	1.40 ± 0.02	1.41 ± 0.04
Mean fetal length (mm)	23.8 ± 0.3	23.7 ± 0.2	24.0 ± 0.2	23.8 ± 0.3

* 1.0 per cent for 5.5 weeks; 0.5 per cent thereafter.

from the reports of Strout *et al.*[15] and Pope and Persaud.[16] Both groups performed limited studies on the reproductive effects of enflurane, administered for eight hours per day, to Sprague-Dawley rats, the former at 11 and 64 ppm, and the latter at 3200 ppm. These studies were negative and help to justify our extrapolation of these data to humans; the more animal species in which essentially negative results occur, the more confident one can be that human exposure is safe, also.

We cannot explain with certainty why we obtained positive results at the anesthetic concentration of enflurane in Experiments A and B, although we can offer several possible reasons for our findings. Premating weight gain (Experiment A) and gestational weight gain (Experiment B) were reduced, so maternal nutritional status may have been adversely affected. Another possible explanation is that hypothermia may have been present during exposure. In our halothane study,[6-7] core temperatures as low as 24° C were measured when mice became anesthetized, and, in fact, only 9 of 23 mice survived the anesthetic dose of halothane. Major temperature reductions would result in significant metabolic, circulatory, and respiratory alterations in the pregnant dams which could lead to fetal abnormalities. Body temperature was not measured in this study but almost certainly it was not as low as in the halothane experiment, since survival of high-dose enflurane mice was not impaired. A third possibility is that enflurane at high concentrations is a mild teratogen. With the exception of studying only one animal species in the teratology portion of the investigation, the present study was carried out in accordance with the guidelines of the U. S. Food and Drug Administration for studies of reproduction.[17] Exposure schedules were designed to detect adverse effects of maternal or paternal exposure during all phases of the reproductive process. Several exposure levels were studied to define a dose-response relationship for any observed effect. Large numbers of mice (443 dams, 4743 fetuses) were examined to increase the probability that an effect, if present, would be detected. 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 enflurane is associated with adverse maternal or paternal reproductive effects, embryotoxicity or teratogenicity. Moreover, there is a wide margin of safety between levels of enflurane that are present in unscavenged operating rooms and those that result in alterations in rodent reproduction and embryonic development.

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