

Fetotoxicity in Rats Following Chronic Exposure to Halothane, Nitrous Oxide, or Methoxyflurane

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An animal model was used to investigate the comparative fetal toxicities of three inhalational anesthetics. Pregnant Sprague-Dawley rats were exposed for eight hours a day throughout the 21 days of gestation to graded concentrations of halothane (0.16–0.32 per cent), or nitrous oxide (1–50 per cent), or a nitrous oxide (10 per cent) and halothane (0.16 per cent) mixture, or methoxyflurane (0.01–0.08 per cent). High subanesthetic concentrations of all the inhalational anesthetics could cause fetal growth retardation (e.g., 3–21 per cent decreases in normal fetal weights), but this was unaccompanied by significant fetal loss (overall rate: 4.8 ± 1.2 per cent, mean \pm SE, in anesthetic groups) or any evidence of skeletal or gross abnormalities related to treatment. It is concluded that these rodent studies do not implicate any specific inhalational anesthetic agent in fetal toxicity, and that the effects of additional factors, such as stress, must be considered. (Key words: Toxicity, fetal, teratogenicity; Anesthetics, volatile, halothane; Anesthetics, volatile, methoxyflurane; Anesthetics, gases, nitrous oxide.)

RECENTLY, the operating room environment has been implicated as a cause of certain adverse effects on pregnant women and their unborn children. Retrospective epidemiologic studies from the U.S.S.R.,¹ Denmark,² the United Kingdom,³ and the United States⁴ have suggested that women who work in the operating room during pregnancy have a greater chance of spontaneous abortion or of having a child with a congenital abnormality than do women engaged in similar pursuits but in different environments.

There is some evidence in animals for⁵⁻⁸ and against⁹ the teratogenicity of clinical concentrations of inhalational anesthetics, but there are few data describing the fetal effects of prolonged exposure to subanesthetic concentrations during pregnancy. We previously studied pregnant rats exposed to a range of subanesthetic concentrations of halothane during the period of maximum fetal development.^{10,11} These studies failed to demonstrate teratogenicity due to the

treatment, but when the rats were exposed daily throughout pregnancy to halothane, 0.16 per cent, some evidence of fetal growth retardation was seen.

In the present work we have further examined the potential of subanesthetic concentrations of halothane to impair fetal growth in the rat. These data have been compared with those obtained using the same test model with subanesthetic concentrations of nitrous oxide, a halothane/nitrous oxide mixture, and methoxyflurane. The aim of these studies was to determine whether these anesthetic agents were capable of evoking fetal toxicity in the rat as a result of daily exposure throughout pregnancy.

Materials and Methods

ANIMALS AND EXPOSURE CHAMBERS

Sprague-Dawley rats were bred under barrier-maintained conditions (specified pathogen-free) in the Comparative Medicine Division at the Clinical Research Centre, fed an autoclaved standard small animal diet (Spillers No. 1 Lab. Animal diet) and given water *ad lib*. Nulliparous females 250–300 g in weight were placed overnight with proven males, and the day vaginal plugs were observed was designated day 1 of pregnancy. These animals have a 21-day period of gestation, with implantation during days 1 to 7 and primary organ system development on days 8–12. The pregnant animals were marked, weighed, and randomly assigned to either a control or an anesthetic-exposure group. Each group consisted of eight to 10 animals. Two 170-l Perspex chambers with external nylon tubing gas-circulating systems were constructed so that standard animal breeding cages could be inserted and removed without the excess stress of transferring animals between cages. All aspects of the environments of the two chambers were maintained as similar as possible except that a controlled level of anesthetic was added to one of them. Ambient temperatures and relative humidities were monitored continually and controlled with internal and external cooling coils. Oxygen and carbon dioxide levels were measured hourly and additional oxygen flow added to maintain the oxygen concentration at 21 ± 0.5 per cent. There was no accumulation of carbon dioxide or excessive humidity. Tests were carried out to ensure that there were

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no gas concentration gradients within the chambers during the periods of exposure.

The anesthetic concentrations were monitored every 10 min throughout the study, using a specially modified automatic gas chromatograph (Pye 104 using a 78 C column of Chromasorb W + 10 per cent OV-101 silicone). After the chamber was closed the concentration increased to the predetermined level within half an hour and was normally maintained within ± 4 per cent of the desired value.

EXPERIMENTAL REGIMEN

The exposure period for the main series of experiments was eight hours a day for the full 21 days of gestation. It was necessary to stagger the start and end of the exposure period, since the average number of pregnant females obtained from the breeding colony was only four/day. The animals in the initial series were allowed food and water *ad lib.* during the period outside the exposure chamber. In the later series of experiments, paired feeding was employed to determine the effect on the control rats of food deprivation alone. Different groups of animals were exposed to a range of subanesthetic concentrations of halothane 0.16–0.32 per cent, or nitrous oxide 1–50 per cent, or methoxyflurane 0.01–0.08 per cent, or a combination of halothane, 0.16 per cent, and nitrous oxide 10 per cent.

In addition to the main series of experiments, an anesthetic-exposure group and a control group of rats were exposed to halothane, 0.32 per cent, for the first eight days of gestation—*i.e.*, during the period of implantation only. In all our experiments care was taken to minimize any stress that the rats might have experienced by being in the exposure chambers. There were four instances when an animal became unavoidably excited and had to be restrained by hand during marking, weighing, or transferring to a clean cage. In these cases the animal was immediately excluded from the experimental or control group and assigned to a separate "stress group." This stress group was subsequently put into a separate cage in the control environmental chamber.

The animals were sacrificed on the last morning of pregnancy and records taken of maternal liver and kidney weights, fetal weight and number, fetal loss, and crown–rump length and sex ratio of the fetuses. Fetal loss was defined as the number of resorption sites and dead fetuses, expressed as a percentage of the total number of conceptuses. All fetuses were routinely examined for signs of gross abnormalities using a hand lens. Representative maternal tissues were fixed for microscopic examination. From each litter,

5 or 6 fetuses were selected and cleared for alizarin red-S staining and skeletal examination.

All our data were analyzed by Student's *t* test using the means and standard errors of the grouped fetal observations for each anesthetic concentration and its appropriate control. There was no significant between-litter variability, which would have necessitated a more complex analytic approach. We deliberately restricted our initial group sizes to eight to ten animals to avoid environmental stress, etc. However, our graded anesthetic concentrations were chosen to be at the upper end of the concentration range in order to allow us also to combine the data for the same anesthetic and reanalyze it with pooled group sizes of approximately 20–30 rats.

Results

FETAL LOSS

Almost all animals included in these studies on the basis of vaginal plug formation were confirmed to be pregnant at post-mortem examination. The occasional nonpregnant rat or the reassignment of an animal to the general "stress group" resulted in minor variation of the numbers of animals in each group. The only exception was one particular set of experiments using halothane, 0.32 per cent, which had a surprisingly low number of pregnancies (one rat in ten). However, additional experiments using the same concentration of halothane and exposing during the period of implantation (days 1–8) produced no evidence of a consistent failure of pregnancy to occur (table 1). We did not repeat the full gestational exposure at the highest concentration because of the ethical problems of imposing severe nutritional stress (see table 3).

In all our experiments with halothane, methoxyflurane, or the halothane/nitrous oxide mixture there was no instance where a significantly higher fetal loss occurred in the anesthetic-exposed animals (table 1). Two concentrations of nitrous oxide (1 and 10 per cent) produced fetal losses significantly different from their specific controls, but these findings were not significant when compared with the overall control levels and the normal spontaneous fetal loss for the rat in our breeding colony (<10 per cent). The highest concentration of nitrous oxide (50 per cent) did not produce a fetal loss significantly different from its own control or from those of the lower concentrations.

The animals reallocated to the general "stress group" over the whole period of our studies had a dramatic increase in fetal loss (65–100 per cent)

TABLE 1. Litter Sizes and Percentage Fetal Losses

Agent	Concentration (v/v per cent)	Anesthetic Group			Control Group		
		Pregnant Rats	Live/Litter (SE)	Per Cent Fetal Loss (SE)	Pregnant Rats	Live/Litter (SE)	Per Cent Fetal Loss (SE)
Halothane	0.16	7	14.0 (1.5)	1.0 (1.8)	6	14.2 (0.8)	0
Halothane	0.32	1	14.0	0	7	14.1 (0.8)	0
Halothane	0.32*	9	14.0 (1.5)	5.3 (3.7)	10	14.7 (0.8)	6.4 (3.3)
Nitrous oxide	1.0	7	12.7 (1.4)	8.2 (3.3)	8	14.3 (0.7)	0
Nitrous oxide	10.0	7	14.3 (0.6)	8.3 (3.7)	8	11.3 (1.3)	1.1 (0.8)
Nitrous oxide	50.0	10	12.0 (0.9)	10.4 (2.2)	10	13.7 (0.6)	8.1 (2.5)
Halothane + nitrous oxide	0.16 10.0	9	13.6 (0.8)	3.2 (1.7)	10	13.4 (0.7)	5.6 (1.9)
Methoxyflurane	0.01	10	15.6 (0.5)	1.3 (1.4)	11	13.0 (1.0)	2.7 (1.0)
Methoxyflurane	0.04	7	14.3 (1.1)	4.8 (2.8)	9	14.2 (0.6)	5.9 (2.5)
Methoxyflurane	0.08	5	13.2 (0.6)	5.7 (1.4)	11	12.5 (0.5)	2.8 (1.1)
"Stress group"		4	1.5 (1.5)	91.3 (8.8)			

* Exposure period: days 1-8 of gestation.

TABLE 2. Fetal and Placental Weights

Agent	Concentration (v/v per cent)	Anesthetic Group			Control Group		
		Total Number of Live Fetuses	Fetal Weight g(SE)	Placental Weight g(SE)	Total Number of Live Fetuses	Fetal Weight g(SE)	Placental Weight g(SE)
Halothane	0.16	98	4.88 (0.07)‡	0.57 (0.01)	85	5.52 (0.05)	0.57 (0.01)
Halothane	0.32	14	4.26 (0.08)‡	0.54 (0.02)	99	5.26 (0.07)	0.53 (0.01)
Halothane	0.32*	126	5.31 (0.05)	0.57 (0.01)	147	5.29 (0.06)	0.60 (0.01)
Nitrous oxide	1	89	5.31 (0.07)	0.51 (0.01)‡	114	5.45 (0.04)	0.59 (0.01)
Nitrous oxide	10	100	4.22 (0.05)‡	0.42 (0.01)†	90	5.02 (0.05)	0.45 (0.01)
Nitrous oxide	50	120	4.35 (0.07)‡	0.43 (0.01)†	137	5.51 (0.04)	0.47 (0.01)
Nitrous oxide + halothane	10 0.16	122	4.66 (0.07)‡	0.56 (0.01)	134	5.77 (0.05)	0.51 (0.01)
Methoxyflurane	0.01	156	5.18 (0.04)‡	0.47 (0.01)	143	5.43 (0.05)	0.45 (0.01)
Methoxyflurane	0.04	100	4.69 (0.08)‡	0.48 (0.01)	128	5.60 (0.05)	0.49 (0.01)
Methoxyflurane	0.08	66	4.47 (0.06)‡	0.43 (0.01)	138	4.90 (0.03)	0.43 (0.01)
"Stress group"		6	3.25 (0.19)	0.31 (0.02)			

* Exposure period: days 1-8 of gestation.

† $P < 0.01$.

‡ $P < 0.001$.

the variability of which was unrelated to their original group assignment.

FETAL GROWTH AND DEVELOPMENT

The principal positive finding was a decrease in the average fetal weights following exposure to the higher subanesthetic concentrations of all the agents throughout pregnancy (table 2). The decreases in fetal weights were accompanied by corresponding decreases in crown-rump lengths and by slight developmental retardation. This was indicated by decreases

in the numbers of centers of ossification in the distal parts of the limbs and in the vertebral column, and by delayed ossification of the sternbrae.

Thus, the numbers of ossification centers in the fetal vertebral columns were a mean of 35.8 ± 0.2 SE (75 fetuses examined) for the nitrous oxide, 50 per cent, group compared with a mean of 36.6 ± 0.2 for 47 fetuses from its control group. The corresponding changes for the other anesthetic groups were statistically insignificant. The reductions in both fore- and hind-limb ossification centers were only significant for the nitrous oxide, 50 per cent,

TABLE 3. Maternal Food Consumption during Pregnancy

Agent	g/g Initial Body Weight		g/g Increase in Weight	
	Exposed	Control	Exposed	Control
Halothane				
0.16 per cent	1.283	1.617	4.759	3.025
0.32 per cent	0.801	1.501	—‡	2.797
0.32 per cent*	1.555	1.550†	3.802	3.314†
Nitrous oxide				
1 per cent	1.393	1.401	3.900	3.559
10 per cent	1.717	1.693	3.338	3.312
50 per cent	1.518	1.743	3.760	3.188
Halothane, 0.16 per cent, plus nitrous oxide, 10 per cent	1.659	1.960	4.061	3.089
Methoxyflurane				
0.01 per cent	1.601	1.514†	3.255	2.941†
0.04 per cent	1.475	1.433†	5.847	3.039†
0.08 per cent	0.985	1.033†	26.231	3.632†

* Exposure period: days 1–8 of gestation.

† Paired feeding carried out during exposure period.

‡ Decrease in overall weight of one pregnant animal.

group (90 and 88 per cent, respectively, of their control values) and for the nitrous oxide/halothane group (89 and 81 per cent of their control values). In the methoxyflurane, 0.04 and 0.08 per cent, groups there were significant reductions in the ossification centers of the metacarpals and phalanges only (89 and 92 per cent of their control values), while in the nitrous oxide, 10 per cent, group the reduction was significant for the metatarsals and phalanges only (88 per cent of its control value).

No gross skeletal anomaly related to exposure to anesthetics was seen. There was a slight, statistically insignificant, increase in the incidence of supplementary ribs, but these were generally in a very rudimentary condition. Thus, the incidence of rudimentary lumbar ribs on one or both sides was a mean of 11.6 ± 1.7 (SE) per cent for all the anesthetic-exposure groups, compared with 8.0 ± 2.1 per cent for their controls. For well-formed lumbar ribs the percentage incidences were 1.8 ± 0.6 for the anesthetic-exposure groups and 1.4 ± 0.6 for the control groups.

There was no consistent or dose-dependent change in the sex ratio of the fetuses. Thus, the male:female ratios ranged from 1:0.97 to 1:1.42 for the anesthetic-exposure groups and from 1:0.76 to 1:1.29 for the control groups.

Maternal food consumption (Table 3) was measured daily for each group of rats and the total consumed during gestation expressed as grams of food/grams initial body weight and as grams of food/gram in-

crease in body weight over the same period—the final body weight being determined at post-mortem examination. In the later experiments paired feeding between the anesthetic and control groups was employed.

Discussion

The influence of environmental agents on the developing fetus may result in impairment of fetal growth, which is manifested as a gross deformity or as retarded growth. When the effect is severe fetal death can occur. In this paper we have separated "fetal loss" from "fetal growth and development" but this is not intended to imply that we believe different pathologic processes are necessarily involved in the three aspects.

No significant increase in fetal loss or change in the average number of implantations per litter was seen with any of the agents we studied. The two lower concentrations of nitrous oxide may be possible exceptions, since their fetal losses were significantly higher than their own controls. However, the absolute magnitudes (approximately 8 per cent) were small, there was no evidence of concentration dependence, and reanalysis, using all the nitrous oxide data pooled together to provide larger group sizes, did not confirm the significance.

Our results are entirely consistent with findings in our previous studies,^{10,11} which used shorter periods of exposure during the period of primary organ system development only. However, our results differ from some of those of earlier investigators.¹² A plausible reason for this is that we attempted to ensure that our animals were subjected to the minimum of stress. Not only were the animals bred and reared under "barrier conditions" but, during exposure, extra care was taken to ensure that the animals' cages were consistently clean and that metabolic wastes were not allowed to accumulate. Any handling or extraneous noise was kept to a minimum. We believe attention to factors such as these is of paramount importance in chronic exposure experiments using enclosed environments. In support of this, we note that in general our control litter sizes were high and fetal losses low.

The evidence of high fetal losses in the animals that had to be reassigned to our general "stress group" is also consistent with this hypothesis. That the variability in this group was unrelated to the original group allocation is not surprising, since the majority of animals were rejected from the study during the initial marking procedure. No further tests were done to clarify the stress problem in our experi-

ment because of the difficulty in quantitating and defining the nonspecific stress to which the animals were exposed, and because of the problems of non-random selection of the original animals.

It might be suggested that the Sprague-Dawley strain of rat was resistant to teratogens. However, other investigators have already used this strain to demonstrate teratogenic effects with anesthetizing concentrations of anesthetics.^{5,6,8} Seasonal and/or diurnal variations have been implicated previously. The inclusion of a control group with each concentration confirmed that there were no seasonal variations in these particular experiments. The earlier data^{6,12} suggest that diurnal variations, where they occur, are associated with increased susceptibility during the day. We therefore chose to expose all our animals at the same time of day in order to facilitate comparisons between different concentrations and different agents.

The average fetal weights were decreased when animals were exposed to high subanesthetic concentrations, and this observation may be due to a number of factors. In some cases the placental weights were significantly decreased, but the fetal/placental weight ratios were variable in both anesthetic-exposure and control groups. The influence of maternal nutrition cannot be ignored, but when paired feeding was employed in the methoxyflurane exposures, the same differences were found to be present. Furthermore, little difference was evident between the average fetal weights in the control groups with and without paired feeding, indicating that reasonable food deprivation does not decrease fetal weight. We cannot exclude the possibility that some type of malabsorption or other defect may prevent adequate nutrition. When expressed as grams food consumed/gram increase in weight, it was seen that the animals exposed to anesthetics ate more food for the same weight increase than did the controls. Indeed, for the highest methoxyflurane concentration the maternal weight increases were so low that the relative food consumption increased dramatically. We would have preferred to have measured the daily food consumption of each individual animal rather than the averaged group data as presented in table 3. However, this would have involved using individual breeding cages (which for our animals would have been an abnormal environment) and imposed the additional stress of daily weighing.

Although the decrease in fetal weight was accompanied by slight developmental retardation, we found no significant increase in either the number and type of skeletal anomalies or the presence of gross deformities. That ultrastructural or other

changes may have been present remains a possibility, although it is surprising that, since they are thought to occur with exposure to 10 ppm halothane,¹³ we failed to observe any manifestation of fetal organ damage using concentrations as high as 3,200 ppm.

In our experiments we deliberately did not search for subtle minor variations, which are difficult to interpret. Instead, we increased the anesthetic concentrations until statistically significant gross changes occurred. This has the obvious disadvantage that, unlike other studies,^{13,14} the levels of exposure in our study were higher than those occurring in operating rooms, but of course the relative susceptibilities of the different animal models are unknown. The upper limits of the inspired anesthetic concentrations chosen were a little more than a third of their MAC levels. This was the "maximum tolerated" subanesthetic dose. At this dose level some of the animals were somnolent during the exposure period but were easily arousable when taken out of the exposure chamber for feeding, etc. The lower limits of our concentration range in this study were 0.007–0.12 MAC. We speculated initially that there might be adverse teratogenic effects associated with very low levels of anesthetics that might not be manifest at higher levels. However, this seems unlikely, particularly because our previous studies of halothane with the same animal model using concentrations as low as approximately 0.001 MAC failed to demonstrate any evidence of teratogenicity.^{10,11} There was evidence that the fetal growth retardation increased with increasing anesthetic concentrations. This was consistent for halothane (including the data for one comparable experiment using halothane, 0.16 per cent, which formed part of our preliminary investigations)¹¹ and for nitrous oxide, but not for the higher concentrations of methoxyflurane.

We also studied the combination of nitrous oxide and halothane at their penultimate dose levels when used alone. There has been some evidence in cellular studies¹⁵ that these agents can have synergistic effects. However, there was no evidence of synergistic fetal growth retardation or of any increased incidence of induced fetal abnormalities at the anesthetic concentrations we used.

The major finding of the experiments reported here was that high subanesthetic concentrations of inhalational anesthetics can cause fetal growth retardation unaccompanied by fetal loss or abnormalities and unrelated to a specific agent. This would suggest that changes apparently due to exposure to subanesthetic concentrations of inhalational agents may well act through a general effect of the anesthetic on the mother and fetus, rather than a directly

toxic effect on the fetus alone. This, coupled with our demonstration that reasonable food deprivation did not decrease fetal weight in our control animals, leads us to speculate that one explanation for the observed retardation might involve a mechanism of redistribution of uterine and fetoplacental blood flows, rather than a specific and directly toxic effect on the fetus itself. We have not been able to test this hypothesis directly, but work in rats has shown that decreasing the uterine circulation impairs fetal growth.¹⁶

These studies with halothane, nitrous oxide, and methoxyflurane do not implicate any specific agent as the cause of the observed retardation of growth. The relative susceptibilities of rodents and man to specific teratogens are indeterminate, and thus discussion of these findings in terms of the human fetotoxicity of absolute concentrations of anesthetics is inappropriate. However, there is evidence that the rat is a suitable animal model for human fetal growth retardation,¹⁶ with the probable exception of brain development.¹⁷ In comparing the different anesthetics, we would suggest that one cannot implicate any specific inhalational agent as the primary health hazard in operating room pollution. It seems likely that other effects, particularly stress or additional atmospheric pollutants, may have produced the abnormalities seen in some of the previous animal studies. This may be due to: a predisposition of the animals by these factors unmasking the deleterious effects of anesthetics; a direct toxicity of the factor itself; or a cumulative effect of all those factors to which animals or people may be exposed. The last explanation would seem the most reasonable, and we would suggest that the cumulative effect of exposure to anesthetic agents, stress in the operating room environment, plus many other factors, is the cause of the abnormalities found in the epidemiologic studies.

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