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Morphologic Changes in Mouse Spermatozoa after Exposure to Inhalational Anesthetics during Early Spermatogenesis

Paul C. Land, M.D., M.Sc., E. L. Owen,† and Harry W. Linde, Ph.D.‡*

The authors studied anesthetic mutagenesis following exposure *in vivo* by use of an adaptation of the mouse spermatozoa morphology assay of Wyrobek and Bruce. The epididymal spermatozoa of (C57Bl/C3H)F_1 mice were examined for morphologic abnormalities following exposure to near-0.1 MAC and greater concentrations of general anesthetics. Twenty exposure hours (4 hr/day × 5 days) were conducted for nitrous oxide, diethyl ether, chloroform, trichloroethylene, halothane, methoxyflurane, enflurane, and isoflurane, each at two concentrations. Twenty-eight days after exposure, epididymal spermatozoa were examined. Statistically significant increases in the percentages of abnormal spermatozoa were found for chloroform, trichloroethylene, and enflurane, compared with controls. These data suggest that direct examination of reproductive cells following exposure to general anesthetics *in vivo* may be useful in the investigation of the genetic toxicities of these compounds. (Key words: Anesthetics, gases: nitrous oxide. Anesthetics, volatile: chloroform; diethyl ether; enflurane; halothane; isoflurane; methoxyflurane; trichloroethylene. Toxicity: mutagenicity; teratogenicity.)

ANESTHETIC-INDUCED genetic damage, particularly in reproductive cells, has been suggested by results of several epidemiologic surveys. Studies conducted in the United States and the United Kingdom have indicated that the operating room environment may be associated with increased risks to workers of spontaneous abortions and congenital abnormalities.[1–3] A possible explanation for these findings could be that an anesthetic causes genetic alterations in reproductive cells. Lethal alterations could produce spontaneous abortion, and those that are not lethal could produce congenital abnormalities.

Laboratory investigation of the genetic toxicities of inhalational anesthetics is essential to evaluate this complex problem. Several types of direct investigation of anesthetic mutagenesis have been reported.[4–7] All of these involve *in vitro* preparations that may fail to duplicate the complex processes of anesthetic distribution and biotransformation. Direct investigation of the genetic toxicity of prolonged exposure to trace concentrations of individual anesthetics *in vivo* has not been practical. An investigation of several agents would necessitate considerable expenditures of time and money, because of the duration of exposure and the numbers of animals involved. An alternative approach to this problem is to examine shorter *in vivo* exposures to higher concentrations of anesthetics in order to determine the potential toxicity of each agent.

We have undertaken a direct investigation of anesthetic-induced genetic toxicity with this alternative approach. The mouse spermatozoa morphology mutagenesis assay of Wyrobek and Bruce[8] was used to examine the genetic toxicity of exposure to inhalational anesthetics *in vivo*. The effects of repeated exposures to near-0.1 MAC and greater concentrations of a number of agents are reported. This initial study was designed to test the utility of the mouse model for investigation of the possible genetic toxicities of inhalational anesthetics.

Materials and Methods

MICE

Male mice, ages 8 to 10 weeks, of genotype (C57Bl × C3H)F_1 were obtained from Cumberland View Farms, Jackson, Tennessee.

Each exposure group of five mice was selected at random from mice of the same age. Members of the same exposure group were caged together throughout the experiment in a suspension wire-bottomed cage. All cages were maintained in a monitored facility with constant temperature and humidity and regulated light–dark cycles. The mice were allowed food (Purina Mouse Chow®) and water *ad libitum*, except during periods of anesthetic exposures, which were initiated when the mice were 11 weeks old.

EXPOSURES

Exposure chambers were constructed from 5-l glass desiccators with fenestrated porcelain floors. Gas entered and left via two glass tubes which were passed through rubber stoppers at the top of each chamber. The tubes were arranged to permit delivery of fresh gas below the floor of the chamber and exhaust near the top. A mercury thermometer was suspended in each chamber to measure temperature.

The anesthetics surveyed and their suppliers are

* Assistant Professor of Anesthesia.
† Senior Research Technician.
‡ Professor of Anesthesia.

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Address reprint requests to Dr. Land.

TABLE 1. Levels of Morphologically Abnormal Epididymal Spermatozoa 28 Days after 4 Hr/Day × 5 Day Exposures to Various Agents

	Concentration (vol per cent)	MAC	Survivors (n)	Per Cent Abnormal Spermatozoa (±SEM)
Air			15	1.42 ± 0.08
Nitrous oxide (a)	80	0.53	5	1.44 ± 0.19
Nitrous oxide	8	0.053	5	1.64 ± 0.15
Diethyl ether (b)	1.6	0.5	5	1.24 ± 0.11
Diethyl ether	0.32	0.1	4	1.70 ± 0.23
Chloroform (b)	0.08	0.1	9	3.48 ± 0.66*
Chloroform	0.04	0.05	9	2.76 ± 0.31*
Trichloroethylene (c)	0.20	1.	10	2.43 ± 0.15*
Trichloroethylene	0.02	0.1	5	1.68 ± 0.17
Fluroxene (a)	3.0, 0.3	1, 0.1	0	—
Methoxyflurane (d)	0.10	0.5	5	1.24 ± 0.17
Methoxyflurane	0.01	0.05	5	1.24 ± 0.05
Halothane (c)	0.80	1	5	1.40 ± 0.18
Halothane	0.08	0.1	4	1.13 ± 0.17
Enflurane (e)	1.20	1	8	2.04 ± 0.13*
Enflurane	0.12	0.1	5	1.50 ± 0.18
Isoflurane (e)	1.00	1	5	1.70 ± 0.18
Isoflurane	0.10	0.1	5	1.20 ± 0.24

Sources: a, Ohio Medical Products, Madison Wisconsin; b, Fisher Scientific Company, Itasca, Illinois (reagent grade); c, Ayerst Laboratories, New York, New York (gift); d, Abbott Laboratories, North Chicago, Illinois; e, Ohio Medical Products, Madison, Wisconsin (gift).
* $P < 0.01$.

listed in table 1. The volatile agents were delivered in air from calibrated vaporizers (Fluomatic, Foregger). Nitrous oxide was administered in oxygen or oxygen plus air to maintain an $F_{I_{O_2}}$ of 0.20. The total flow of fresh gas to each chamber during exposure was 2.5 l/min.

Each cage of five mice constituted an exposure group. Exposures were conducted 4 hr/day (at 1000 to 1400 hr) for five consecutive days. Chamber concentrations of approximately 1 MAC and 0.1 MAC were originally planned for each agent. The higher concentrations were adjusted and the exposures were repeated for those agents that produced mortality rates of more than 20 per cent at 1 MAC. Each exposure was followed by a recovery period of one hour in air before the animals were returned to their cages. Three control groups were included at separate times during the study. These animals were exposed to air under conditions identical to those maintained for the test groups.

The anesthetic concentration of the delivered gas and the CO_2 concentration of the exhaust gas were monitored periodically by gas chromatography. The chamber temperatures were measured directly.

PREPARATION AND EVALUATION OF SPERMATOZOA

The methods of preparation and evaluation of spermatozoa were adapted from those originally described by Wyrobek and Bruce.[8] Mice were sacrificed by cervical dislocation 28 days after the first day of exposure. Following sacrifice, both cauda epididymides were removed. These were minced with scissors into 2 ml physiologic buffered saline solution, pipetted, and filtered through stainless gauze. The filtered suspension was mixed 10:1 with 1 per cent eosin Y in H_2O and stained overnight at 4°C. Duplicate, labelled slides were prepared and mounted under coverslips. The labels were covered with tape, and the slides were mixed and coded.

To avoid bias by the examiner, the code was broken only after all of the slides had been evaluated for morphologic abnormalities of spermatozoa.

All slides were examined microscopically at 400× magnification with green filters. A thousand spermatozoa on each slide were evaluated for morphologic abnormalities, according to published criteria.

Preparations from 20 per cent of the mice were selected and examined independently by a second investigator. The numbers of morphologically abnormal cells were recorded in percentages.

STATISTICS

The mean percentage abnormal spermatozoa was calculated for each exposure group. Each was compared with the mean percentage abnormalities for the single control group of 15 mice by the test for two samples and by a one-way analysis of variance (F test).

Results

The anesthetic concentration delivered was maintained within 5 per cent of the intended concentration for each agent. The CO_2 concentration of the exhaust gas was maintained below 0.3 per cent, and the chamber temperature was 20° ± 1° C during all exposures.

Repeated exposures to methoxyflurane, chloroform, and diethyl ether at 1 MAC produced mortality rates of more than 20 per cent. Lower exposure concentrations were chosen for these agents (table 1), and the exposures were repeated. Exposures to 1 MAC and 0.1 MAC fluroxene produced mortality rates of 100 per cent, and investigation of this agent was abandoned.

The percentages of morphologically abnormal spermatozoa following exposures to the individual agents are shown in table 1. No increase in abnormal forms was found for nitrous oxide, diethyl ether, halothane, methoxyflurane, or isoflurane exposures at either concentration.

Statistically significant increases of abnormal spermatozoa resulted from repeated exposures to 1 MAC trichloroethylene or enflurane or either concentration of chloroform. These exposures were repeated with additional groups of five mice with the same results. The data from both exposure groups for each agent were pooled for statistical comparisons.

Examples of the spermatozoal abnormalities present following exposures to enflurane are shown in figure 1. These forms are similar to those found after exposures to trichloroethylene and chloroform.

The variation of the percentage abnormalities was less than 0.03 per cent between duplicate slides and less than 0.12 per cent between examiners.

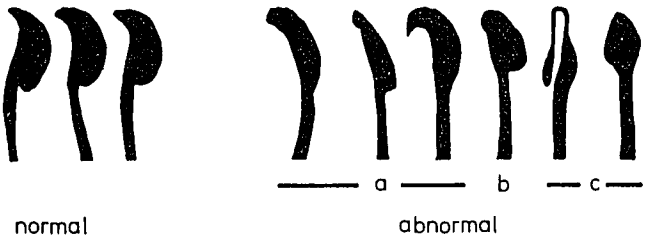

FIG. 1. Forms of normal and abnormal spermatozoa after 20 hours of exposure to 1 MAC enflurane. Normal forms (*left*) have constant dimensions, with characteristic hooks and tail attachments. Abnormal forms (*right*) may be flattened (*a*), have abnormal hooks (*b*), or be amorphous (*c*). Counts of abnormal forms were not subdivided. Figures were traced from photomicrographs at 400× magnification.

Discussion

The clinical significance of the increases of abnormal spermatozoa in mice following exposures to chloroform, trichloroethylene, and enflurane cannot be evaluated. However, direct investigations of this type could be ultimately beneficial for investigating the question of heritable genetic damage resulting from exposure to inhalational anesthetics. The mouse spermatozoa morphology assay has been used as an *in vivo* test for environmental mutagens. The sensitivity of this assay has compared favorably with sensitivities of several currently accepted *in vitro* and *in vivo* mutagenesis assays.[9] This suggests that the morphologically abnormal spermatozoa are the results of genetic damage that occurred during spermatogenesis; however, a causal relationship has not been established. If spermatozoal abnormalities are caused by mutations, then the effects of trichloroethylene and chloroform exposures were predictable. Both agents are animal carcinogens,[10,11] and this effect is consistent with the hypothesis of induced genetic damage in susceptible cells. The effect of enflurane on sperm was not predictable. This agent has not been shown to be a carcinogen, and no reproductive toxicity has been reported for it. More extensive investigation of the mechanisms of action of these agents during spermatogenesis is necessary before the results of this study can be interpreted in relation to anesthetic-induced genetic toxicity.

The lack of effect of some of the other anesthetics on spermatozoal form is also important. Both halothane and nitrous oxide have been shown in experimental animals to be toxic to male reproductive cells. Kripke *et al.* found reversible atrophy of the mouse seminiferous tubule following both continuous and intermittent exposures to 20 per cent nitrous oxide for 35 days.[12] We have found that intermittent exposure to 80 per cent nitrous oxide for five days has no significant effect on production of spermatozoa in mice (unpublished data). Coate *et al.* reported dose-related increases of chromosomal abnormalities in rat spermatogonia following prolonged exposure to trace concentrations of nitrous oxide plus halothane in air.[13] If the chromosomal response also involves mutations in the spermatogonia, it should be detectable with the spermatozoa morphology assay. Our data indicate that neither of these agents is mutagenic with *in vivo* exposure. However, further investigation of anesthetic action in the testes is indicated to increase our understanding of these important processes.

In this study, an incubation period of 28 days was used, rather than the 35 days described by Wyrobek and Bruce. This alteration was designed to evaluate the mid-cycle of spermatogenesis, rather than to precisely localize a portion of the cycle. Exposure during the broader range of events occurring during the later stages of cell division was intended to compensate for any possible alterations of cell division that have been reported for some anesthetics. An incubation period of 28 days should have allowed the maturation of type B spermatocytes which were in meiosis during the exposure period. Without measuring the duration of spermatogenesis following each anesthetic exposure, however, the exact site of toxicity is unclear. Even if some of these agents inhibit meiosis, the cytotoxic effects of anesthetic exposure must have occurred during either the first or the second meiosis, or during the period of maturation and migration.

The cycle of spermatogenesis in the mouse normally lasts 35–36 days. This process has been described by Clermont[14] to contain five phases. In each phase the cells have characteristic morphologic features. Every nine days, a population of diploid stem cells (spermatogonia) undergoes mitosis to produce type A spermatocytes. The formation of this group of cells marks the beginning of the cycle (day 0). During the next seven days, type A spermatocytes undergo five mitoses to produce a population of type B spermatocytes. During the next 14 days (days 7–21), the diploid spermatocytes undergo first and second meioses to form haploid secondary spermatocytes, then spermatids. The spermatids mature in the seminiferous tubule into spermatozoa, with characteristic acrosomal caps, condensed chromatin, and tails. The spermatozoa are released into the seminiferous tubule, and they migrate to the epididymis. Maturation and migration are completed in 14 days (days 21–35).

Several features of spermatogenesis indicate the utility of this model for direct investigation of genetic toxicity. First, a single population of cells passes through each phase of the cycle simultaneously. Thus, reasonable certainty exists that all of the epididymal spermatozoa obtained for morphologic evaluation were at the same developmental phase in the testes during the periods of anesthetic exposure. The physiologic factors that govern the susceptibility of the individual cell to toxins should not vary significantly among cells. Therefore, the possibility of false-negative responses, caused by dilution of the sample with unresponsive cells, is minimized.

A second feature is the almost constant normality of epididymal spermatozoa from (C57Bl × C3H)F_1 mice, plus the low percentage of morphologic abnormalities in the control group. Wyrobek and Bruce reported 1.8 per cent abnormal forms for a control group composed of two different F_1 hybrids. Our control value of 1.42 per cent for a single hybrid is consistent with those findings. These low values permit the use of small samples of mice for each agent. With this model, comparisons of the effects of several agents can be accomplished economically.

The ultimate usefulness of the mouse spermatozoa assay is suggested by comparisons of our results with those of mutagenesis assays *in vitro*. Baden *et al.* found that, among the agents included in this study, only trichloroethylene was weakly mutagenic to *Salmonella typhimurium*.[4–6] White *et al.* found no increase of sister chromatid exchanges in Chinese hamster oocytes following exposures to agents included in this study.[7] Our different results following exposures *in vivo* could have been due to many factors, including testicular uptake and biotransformation of anesthetics and accumulation of toxic metabolites produced elsewhere. The intracellular concentrations of toxic metabolites following exposure *in vivo* and recovery could exceed those obtained by equilibration of an *in vitro* system with an anesthetic-containing atmosphere. This view is supported by Waskell's report that relatively large amounts of chloral hydrate, which is a metabolite of trichloroethylene, are necessary to induce mutations in *Salmonella*.[15] It is also supported by Cohen's observation of high concentrations of nonvolatile ^{14}C halothane metabolites in the testes of heart donors.[16]

We have examined current, future, and obsolete anesthetic agents to determine their potentials for damaging reproductive cells when administered repeatedly at near-clinical concentrations. Only chloroform, trichloroethylene and enflurane produced morphologic alterations of spermatozoa that may have indicated genetic damage. The anesthetic concentrations used for this study did not duplicate the usual conditions encountered by either surgical patients or operating room workers. However, the results of the study suggest that the mouse model may be useful for investigating the question of anesthetic-induced genetic damage.

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