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Mutagenicity of Inhaled Anesthetics in Drosophila melanogaster

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The mutagenic effects of several inhaled anesthetic agents were investigated using the sex-linked recessive lethal assay in the fruit fly, *Drosophila melanogaster*. Male wild-type flies were exposed for 1 hr to either halothane, enflurane, isoflurane, or fluroxene at vapor concentrations of 1 or 2% or to nitrous oxide at concentrations of 40 or 80%. Control flies were exposed to air alone. Following treatment, male flies were mated with untreated virgin females of the *Basc* strain and the rate of sex-linked recessive lethals was determined in the F_2 generation. Halothane and fluroxene produced a dose-dependent and statistically significant increase in the rate of sex-linked recessive lethals, whereas enflurane, isoflurane, and nitrous oxide were not mutagenic at the concentrations tested. (Key words: Anesthetics, gases: nitrous oxide. Anesthetics, volatile: enflurane; fluroxene; halothane; isoflurane. Toxicity: mutagenicity.)

MANY INVESTIGATORS have examined toxicity of inhaled anesthetics and, in particular, have studied their mutagenic potential in a variety of test systems.[1] Most tests have shown that currently used inhaled anesthetics, nitrous oxide, halothane, enflurane, and isoflurane, are not mutagenic, whereas previously used anesthetics such as fluroxene, which contain the vinyl moiety, are mutagenic. However, several studies have been inconsistent with these general findings. For example, both halothane and nitrous oxide have been reported to slightly increase the rate of sex-linked recessive lethals in the fruit fly, *Drosophila melanogaster*[2,3]; enflurane, isoflurane, and fluroxene have not been tested in this system. Because of the marginally positive results with halothane and nitrous oxide and the lack of information with the other anesthetics, we undertook to examine all these agents for their ability to induce sex-linked recessive lethals in Drosophila.

The advantages of using Drosophila are numerous. This species is capable of absorbing, circulating, metabolizing, and excreting chemicals while economically producing large numbers of offspring in a relatively short period of time.[4] In addition, the fruit fly has a short life history of about 9–10 days when reared at 25° C. Furthermore, besides humans, the genetics of this organism is best known. The sex-linked recessive lethal test has been used in this study because it provides a short-term *in vivo* mutagen screening system employing a eukaryotic organism.[5] The test measures the frequency of lethal mutations in approximately one-fifth the total genome of the organism.

Materials and Methods

The methods essentially were those of Würgler *et al.*[5] In brief, two strains of *Drosophila melanogaster*, the Oregon K, a wild-type, and *Basc*, a marker stock, were used in this study to test for recessive sex-linked lethality. This marker, *Basc*, which is an acronym for Bar, apricot, and scute, carries a dominant *B* (Bar-eyed) gene, which gives narrow eyes in a homozygous or hemizygous condition, and kidney-shaped eyes in a heterozygous condition. The gene w^a (white apricot) is recessive and gives an apricot-eyed phenotype in the homozygous condition. In addition, *Basc* chromosome contains the recessive gene *sc* (scute), which when visible affects the form and number of certain bristles.[6] Male wild-type flies, 2–3 days old, were exposed to an acute dose of either halothane, enflurane, isoflurane, or fluroxene at vapor concentration of 1 or 2%. For nitrous oxide, the concentrations used were 40 or 80%. These doses, which are within the clinical range, were found not to affect survival in preliminary LD_{50} studies.[7] Control flies were exposed to room air alone. All exposures were for 1 h at 25 ± 1° C. The relative humidity was maintained at 50 ± 5%. During exposure, the concentration of oxygen was monitored by an IL 402 oxygen monitor (Instrumental Laboratory, Lexington, Massachusetts) and maintained at 21 ± 1%. Anesthetic concentrations were measured at the start and end of exposure using gas chromatography or IR analysis and did not decrease by more than 2% during exposure.

Immediately following exposure, 30 male flies from each treatment group were selected at random. Each male was individually mated with three untreated virgin female flies of the *Basc* strain in individual vials with fresh medium (Carolina Biological Supply, Burlington,

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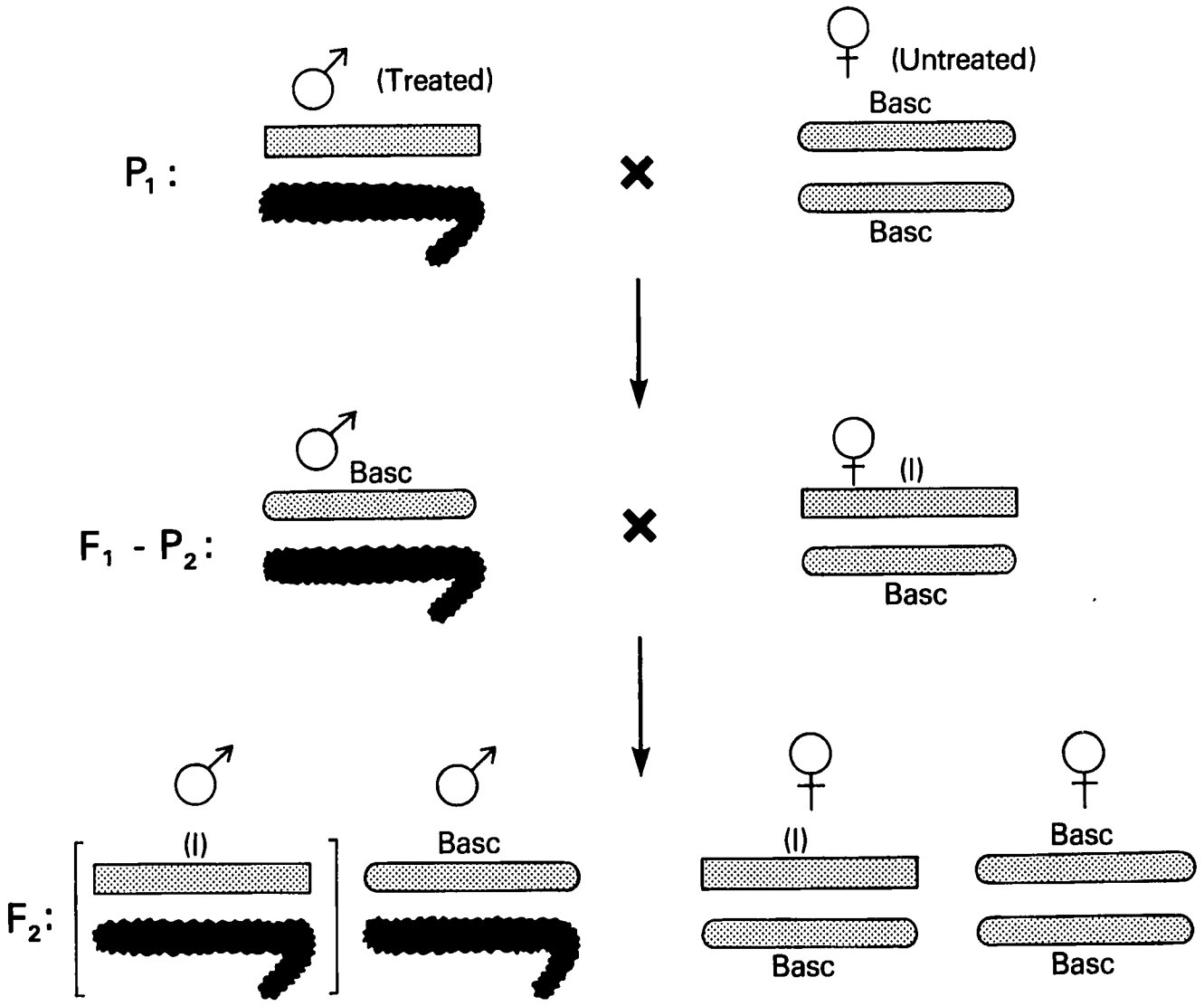

FIG. 1. Cross of wild-type treated male fly with *Basc* untreated virgin female fly for induction of sex-linked recessive lethals in Drosophila. Absence of wild-type male files in the second generation of flies (F_2) indicates lethality.

North Carolina). The mating scheme is depicted in figure 1. Three days after mating and after the eggs were deposited, female flies were discarded and male flies immediately were mated individually with three fresh *Basc* females in new vials to produce the second brood. Three days later, the mating procedure was repeated to produce the third brood. Thus, the mutagenic activity of the anesthetics on germ cells at different stages (spermatozoa, spermatids, and spermatocytes) was tested.[5]

The F_1 (first filial generation) flies were collected for 2–3 days to ensure adequate numbers and sexual maturity. The F_1 female flies with kidney-shaped red eyes then were mated individually with three male flies of *Basc* phenotype, that is, to their brothers, in individual vials. Twenty or more F_1 female flies were mated from each of the 30 P_1 (first parental generation) treated male flies, for a total of at least 600 F_1–P_2 (first filial–second parental generation) female flies per brood (table 1). The F_1–P_2 flies were removed from the vials after 5 days, and all cultures were kept for 12–14 days to guarantee that male flies with semilethal mutations characterized by a longer development hatched. The other broods were similarly mated. The F_2 (second filial generation) progeny for each brood was examined using a dissecting microscope over a 10-day period, beginning with day 1 of eclosion (emergence of fly from pupa), to determine the occurrence of sex-linked recessive lethals. The absence of wild-type males indicated lethality. Only cultures with 40 or more progeny (males and females together) were included. If 40 or more progeny are present, and no wild-type male flies are observed, the

TABLE 1. Induction of Sex-linked Recessive Lethals Following Exposure of Male *Drosophila melanogaster* to Various Anesthetics for One Hour

Anesthetic Agents	Amount Used (% v/v)	Brood	Days Posttreatment	No. Chromo. Tested	No. Lethals Detected	Per Cent Lethals Calculated
Control	0	I	1–3	602	1	0.166
		II	4–6	597	2	0.335
		III	7–9	589	1	0.170
		I–III	1–9	1,788	4	0.224
Halothane	1	I	1–3	600	7	1.160*
		II	4–6	579	5	0.864
		III	7–9	581	8	1.376*
		I–III	1–9	1,760	20	1.136†
	2	I	1–3	601	11	1.830*
		II	4–6	607	8	1.318
		III	7–9	610	9	1.472*
		I–III	1–9	1,818	28	1.539†
Fluroxene	1	I	1–3	600	3	0.500
		II	4–6	595	1	0.168
		III	7–9	597	8	1.340*
		I–III	1–9	1,792	12	0.670*
	2	I	1–3	628	1	0.159
		II	4–6	604	4	0.662
		III	7–9	611	9	1.720*
		I–III	1–9	1,843	14	0.760*
Enflurane	1	I	1–3	607	3	0.494
		II	4–6	610	2	0.327
		III	7–9	598	0	0.000
		I–III	1–9	1,815	5	0.275
	2	I	1–3	591	0	0.000
		II	4–6	607	2	0.329
		III	7–9	610	1	0.164
		I–III	1–9	1,808	3	0.166
Isoflurane	1	I	1–3	591	2	0.338
		II	4–6	605	1	0.165
		III	7–9	601	2	0.332
		I–III	1–9	1,797	5	0.278
	2	I	1–3	599	3	0.501
		II	4–6	598	1	0.167
		III	7–9	604	2	0.331
		I–III	1–9	1,801	6	0.321
Nitrous Oxide	40	I	1–3	610	2	0.330
		II	4–6	607	3	0.494
		III	7–9	603	0	0.000
		I–III	1–9	1,820	5	0.274
	80	I	1–3	605	2	0.331
		II	4–6	597	3	0.503
		III	7–9	593	0	0.000
		I–III	1–9	1,795	5	0.279

* $P < 0.05$.

† $P < 0.01$.

culture can be considered as indeed containing a lethal. The chance of missing a lethal mutation is only $(1/2)^{10}$; that is much less than 1 in 100. After all vials had been examined for lethals, the number of sterile cultures were eliminated from the total number of cultures. The frequency of lethals then was calculated by dividing the number of lethals found in each brood by the total number of tested X-chromosomes in each brood. A cluster of mutations arising from spontaneous mutation in a gonial cell which then replicated was regarded as a single mutation effect. The Kastenbaum-Bowman test,[8] which is a modified version of the Fisher's exact test, was used to compare the mutation rate in each brood of each anesthetic with that of the untreated controls. An increase of twice the spontaneous frequency in conjunction with a dose–response relation is considered a positive response.[6] $P < 0.05$ was considered significant.

Results

Control wild-type flies exposed to room air alone produced only one or two lethals per brood. Total lethality rate was four out of 1,788 gametes tested or 0.224% (table 1).

Both halothane and fluroxene produced a dose-dependent increase in the rate of lethal mutations above

control. At 2% vapor concentration, the highest concentration tested, halothane produced about a sevenfold increase over control values in total lethality rate ($P < 0.01$), whereas fluroxene produced about a 3.5-fold increase ($P < 0.05$) (table 1). Brooding pattern analysis showed that all stages of germ cell development appeared to be equally sensitive to the mutagenic effects of halothane and fluroxene.

Enflurane, isoflurane, and nitrous oxide were not mutagenic at any concentration tested (table 1).

Discussion

In the present study, we found that the spontaneous rate of sex-linked recessive lethal mutations of *Drosophila melanogaster* was 0.224%, a value that is within the normal range of 0.21–0.35% found by other investigators.[2,3,9]

Exposure to 2% halothane for 1 h increased the rate of spontaneous mutation sevenfold. This volatile anesthetic still is one of the most widely used in clinical practice. In a previous study, Kramers and Burm,[3] using the same test system but different dosages, observed a weak mutagenic response with halothane. They exposed flies to 1,000 or 1,600 ppm (v/v) for 14 days, or to 2,100 or 10,000 ppm for 1 or 2 days, dosages many times greater than those used in our study. Halothane also has been reported to cause other types of genotoxicity in Drosophila. Clements and Todd[10] observed that when virgin female flies were fed a solution of 0.345% halothane in 0.1% sucrose for 24 h and were mated with untreated male flies, an increase in disomic and nullosomic eggs was seen. They suggested that halothane caused nondisjunction by interfering with the spindle mechanism of cell division.

Collectively, the studies in Drosophila provide strong evidence that halothane is mutagenic in this species, even at comparatively low dosages. However, halothane has not been shown to be mutagenic in any other test system. In particular, it is not mutagenic in the Salmonella/microsome system under a wide variety of test conditions and anesthetic concentrations.[11–13] The most likely explanation for the different results among the tests is differences in pathways and extent of metabolism. Nonetheless, the disparity in test results casts doubt on the significance for humans of the positive results in Drosophila, especially as several *in vivo* tests in mammals also have been negative.[14]

Fluroxene, an anesthetic that now is seldom used, also showed a dose-dependent increase in mutation frequency in Drosophila. Although at equal vapor concentrations, fluroxene was less mutagenic than halothane, it is about 4.5 times less potent. Thus, it seems likely that equipotent concentrations of fluroxene would be more mutagenic than halothane. In contrast to halothane, all other systems that have been used to test fluroxene for mutagenicity have given positive results. Baden *et al.*[15] found fluroxene to be mutagenic in the Ames' Salmonella system. A dose-dependent mutagenic response occurred at vapor concentrations between 3 and 30%. White *et al.*[16] reported an increase in sister chromatid exchanges (SCE) in Chinese hamster ovary cells following exposure for 1 h at 2.47% vapor concentration. Thus, as with many compounds that contain the vinyl moiety, fluroxene is mutagenic and may be potentially carcinogenic and teratogenic.

Nitrous oxide was not mutagenic in the Drosophila test system in this study. Of numerous studies using many test systems, only one has given marginally positive findings. Garrett and Fuerst[2] demonstrated that nitrous oxide increased the number of recessive sex-linked lethal mutation in Drosophila. Flies gassed in a 100-ml flask for 6 min to 22 ml/min nitrous oxide had a lethal mutation rate of 2.82 ± 0.03, a value significantly greater than the control value of 0.21%. However, the imposition of the hypoxic gas mixture raises an uncertainty about the mutagenic response. The lack of mutagenicity of nitrous oxide seen in our study and in many other studies suggests that nitrous oxide has no mutagenic potential.

The anesthetics agents enflurane and its isomer, isoflurane, which are alpha halo-ethers, were not mutagenic in Drosophila. Our negative findings are in agreement with many mutagenicity studies using other test systems.[16–18]

The concentrations of 1 or 2% for all the volatile anesthetics were selected because they are in the clinical range and are well below the LD_{50} in Drosophila.[7] For nitrous oxide, 80% was chosen as it is the highest concentration that can be delivered in a nonhypoxic mixture. Whether these concentrations are equipotent in Drosophila is unknown and would be difficult to determine.

Many factors must be considered before assessing the risk of these inhalational anesthetic agents to humans. Species variations in drug metabolism and toxicity, different dosages and exposure times, and hypoxia during treatment are some of the factors that make it difficult to extrapolate results from one species to another and from *in vitro* experiments to *in vivo* situations in humans. Nonetheless, knowledge that fluroxene is mutagenic in a number of short-term tests and halothane is weakly mutagenic in Drosophila should be kept in mind when assessing the overall toxicity of these anesthetics in humans.

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