

Lack of Mutagenic Effects of Halothane in Mammals In Vivo

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Rodents were exposed *in vivo* to various clinical doses of halothane to observe structural chromosome aberrations, micronuclei, sister chromatid exchanges (SCEs), dominant lethal mutations, and interferences with phases of the cell cycle. The frequency of chromosome aberrations in bone marrow cells was not increased after exposing Chinese hamsters to 1 per cent halothane once for 3 h, twice for 3 h (exposures 24 h apart), or to 0.5 per cent for 24 h. The analysis of SCEs in bone marrow cells was also negative after exposing hamsters to 1 per cent halothane for 3 h, or to 0.5 per cent for 12 h. In halothane-exposed female mice (0.75 per cent for 16 h), oocyte maturation (meiotic stages) was delayed. Another group of exposed female mice (1 per cent for 5 h) were mated with untreated males. The number of dead implants was not increased as compared to controls. In liver cells of 16-day-old living embryos, neither monosomic nor trisomic cells were observed. Male mice were exposed to 1 per cent halothane for 1 h per day for 48 days. On the evening of the forty-eighth day, they were mated with untreated females. Dominant lethal mutations were not increased as compared to controls. In erythrocytes of these chronically exposed male mice, the frequency of micronuclei was not significantly enhanced. The authors conclude that halothane does not induce mutations under the *in vivo* conditions tested in this study. (Key words: Anesthetics, volatile; halothane. Toxicity: mutagenicity.)

IN A STUDY sponsored by the American Society of Anesthesiologists,¹ it was reported that female members exposed to inhalation anesthetics in operating rooms were subject to an increased risk of cancer, spontaneous abortion, and abnormalities in their children. An increased risk of congenital abnormalities was also reported among the unexposed wives of male operating room personnel. These data led to the assumption that inhalation anesthetics may induce mutations. This hypothesis was tested in the present study, performing experiments with mammals *in vivo*.

Material and Methods

The experiments were performed using 10- to 15-week-old Chinese hamsters (*Cricetulus griseus*,‡ weighing 30 g, and mice of the strain NMRI,‡ 10 to 16 weeks old. The animals were kept at 22° C at a humidity of 50 to 70 per cent and a dark period from

7 P.M. to 6 A.M. A calibrated vaporizer (Dräger AG) was used to deliver halothane (Hoechst AG). The vaporizer was installed into the fresh gas line (air, metered from the compressed air taps in the laboratory), leading to the inhalation chamber. The gas was conveyed through the inhalation chamber, made of Plexiglas® and measuring 20 × 20 × 20 cm. The fresh gas inlet was on the bottom of the chamber, the outlet at the lid of the box and diagonally opposite to the inlet. The total flow of the halothane-air mixture was 1 l/min. In each experiment there was a treatment group which received 0.5 per cent, 0.75 per cent, or 1 per cent v/v halothane in air for a specified time (see below). These dose levels for halothane were selected on the basis of toxicity studies. Groups of four animals were exposed to concentrations up to 2 per cent halothane in air. The maximum tolerable dose (MTD) depends on both animal species and time of exposure. The MTD in Chinese hamsters was 1 per cent halothane in air when exposed for 3 h, and 0.5 per cent when exposed for 24 h. Female mice could be exposed to 1 per cent halothane for 5 h or to 0.75 per cent for 16 h. No mortality occurs in these groups, whereas higher doses were toxic. Negative control animals were exposed to a continuous flow of air only. Positive control animals were exposed to 1.25 per cent vinyl chloride in air for 12 h or to 5 per cent for 24 h.²

TEST FOR STRUCTURAL CHROMOSOME ABERRATIONS

Chinese hamsters were exposed to 1 per cent halothane for 3 h or twice for 3 h, 24 h between exposures. A third group was exposed to 0.5 per cent halothane for 24 h. Twenty-four hours after the beginning of exposure, or 24 h after beginning the second exposure, the hamsters received an intraperitoneal injection of colchicine, 8 mg/kg. Bone marrow chromosomes were prepared 2 h later according to Boller and Schmid.³

TEST FOR INDUCED SISTER CHROMATID EXCHANGES (SCEs)

Chinese hamsters were exposed to 1 per cent halothane for 3 h or to 0.5 per cent for 12 h. The BrdU-tablet method⁴ was used to obtain *in vivo* induced SCEs. A 50-mg BrdU-tablet (Boehringer) was implanted subcutaneously into the neck of hamsters weighing 30 g immediately before starting the halothane exposure. Colchicine was injected 24 h later

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Received from the Institute of Human Genetics and Anthropology, University Düsseldorf, Universitätsstr.1, Gebäude 23.12, D-4000 Düsseldorf, FRG. Accepted for publication January 22, 1981. Supported by the Bundesministerium für Forschung und Technologie, FRG. Contract No. CMT 16.

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‡ Animals were supplied from the "Zentralinstitut für Versuchstierkunde", Hannover, FRG.

TABLE 1. Chromosome Aberrations in Bone Marrow Cells of Chinese Hamsters

Series	Number of Hamsters	Number of Metaphases	Metaphases with Aberrations				G	Number of Metaphases with			
			Including Gaps		Excluding Gaps						
			n	Per Cent	n	Per Cent		B + F	D	E	MA
Negative control	10	1,000	10	1.0	5	0.5	5	4	1	0	0
1 per cent halothane 1 × 3 hours	10	1,000	13	1.3	7	0.7	6	7	0	0	0
1 per cent halothane 2 × 3 hours	10	1,000	12	1.2	8	0.8	4	7	1	0	0
0.5 per cent halothane 1 × 24 hours	10	1,000	16	1.6	8	0.8	8	8	0	0	0
Positive control 5 per cent vinyl chloride 1 × 24 hours	10	1,000	257	25.7*	225	22.5*	32	87	9	76	53

G = gap, B = break, F = fragment, D = deletion, E = exchange figure, MA = multiple aberration.

* $P < 0.02$.

and bone marrow chromosomes prepared 2 h later. Chromosomes were stained using the FPG techniques.⁵

OOCYTE TEST

Female mice were pretreated with hormones to induce ovulation.⁶ Immediately after injection of the luteinizing hormone human chorionic gonadotrophin (HCG), the animals were exposed to 0.75 per cent halothane for 16 h. Thereafter, oocytes were prepared according to Röhrborn and Hansmann.⁶

DOMINANT LETHAL TEST AND ANALYSIS OF EMBRYONIC LIVER CELLS

Female mice, not pretreated with hormones, were exposed to 1 per cent halothane from 10 P.M. to 3 A.M. and mated immediately thereafter with untreated males. In the morning the females were examined, and those with vaginal plugs were assumed to have mated successfully. The uterus content was analyzed for induced dominant lethal mutations at day 16 postconception (p.c.). The livers of the living embryos were removed, cytologically prepared according to Datta *et al.*,⁷ and analyzed for numerical aberrations.

Male mice, 8 weeks old, were exposed to 1 per cent halothane for 1 h per day for 48 days. On the evening of the forty-eighth day they were mated with untreated females followed by the dominant lethal test. In both series the mode of mating was 1:1.

THE MICRONUCLEUS TEST

The bone marrow of the male mice exposed for 48 days was prepared and analyzed for induced micronuclei⁸ 24 h after the last halothane exposure.

STATISTICS

The significance of differences in the yield of metaphases with aberrations and the mutation frequencies in the micronucleus test was verified using the χ^2 test. The data from the SCE test were statistically analyzed using Student's unpaired *t* test. Mutagenicity was considered significant if $P < 0.02$, and the frequency of mutations was > 1.5 -fold compared to the controls. The formula of Ehling *et al.*⁹ was used for the calculation of the percentage of dominant lethal test factors. The number of dead implants per dam was analyzed by Student's unpaired *t* test.

RESULTS

Vinyl chloride, used as a positive control, induced structural chromosome aberrations (table 1) and SCEs (table 2). The percentage of metaphases with aberrations increased from 0.5 per cent in the controls to 22.5 per cent (5 per cent vinyl chloride, 24 h), and the number of SCEs per cell from 3.66 to 17.51 (1.25 per

TABLE 2. In Vivo-induced SCEs in Bone Marrow Cells in Chinese Hamsters

Series	Number of Hamsters	Number of Metaphases	Number of SCEs	Number of SCEs/cell*
Negative control	10	500	1,830	3.66 ± 0.26
1 per cent halothane, 3 hours	10	500	1,997	3.99 ± 0.97
0.5 per cent halothane, 12 hours	10	500	2,258	4.52 ± 0.66
Positive control 1.25 per cent vinyl chloride, 12 hours	4	200	3,502	17.52† ± 1.55

* Values are means ± SD.

† $P < 0.02$.

TABLE 3. Analysis of Oocytes of Mice 16 Hours After the Injection of Human Chorionic Gonadotropin

Series	Number of Mice	Number of Analyzed Oocytes	Oocytes at Metaphase I		Oocytes at Metaphase II	
			Number	Per Cent	Number	Per Cent
Controls	100	227	3	1.3	224	98.7
0.75 per cent halothane 16 hours	100	126	11*	8.7	105	91.3

* $P < 0.02$.

cent vinyl chloride, 12 h). In contrast to the positive control neither the frequency of structural chromosome aberrations (table 1), nor the number of SCEs per cell (table 2) was significantly increased, and/or >1.5-fold compared to the negative controls after exposing hamsters to halothane.

In unexposed females pretreated with hormones to stimulate ovulation, most of the oocytes were at the metaphase II stage; only 1.3 per cent oocytes were still at the metaphase I stage (table 3). In halothane-exposed females the oocyte maturation, *i.e.*, the cell cycle, was delayed. Thus, 8.7 per cent of the oocytes were still at the metaphase I stage instead of metaphase II 16 h after the injection of HCG.

In female mice not pretreated with hormones, exposed to 1 per cent halothane, and mated with untreated males, dominant lethal mutations were not induced (table 4). The number of implants (living and dead) per dam was not decreased and the number of dead implants was not increased, as compared to controls. Furthermore, embryonic liver cells were cytologically examined for aneuploidies. Hyper- or hypoploidies did not occur in either controls or in 16-day-old living embryos of halothane-exposed females (table 5).

A lack of mutagenic effects was also observed in chronically exposed male mice. Neither the frequency of micronuclei in erythrocytes (table 6) nor of dominant lethal mutations (table 4) were significantly increased above the controls.

Discussion

In recent years, mutagenicity studies have been initiated because of epidemiologic surveys (for review

see Baden and Simmon¹⁰), which suggested an increased incidence of malignancies in operating room personnel exposed to inhalation anesthetics.¹ In most of these laboratory investigations performed with microorganisms or cell cultures, halothane was not mutagenic. For example, when halothane was tested in the *Salmonella*/microsome test, no significant increase in numbers of mutations were found.¹¹ In addition to the *Salmonella* assay,¹² halothane was also tested with negative results, using the *Escherichia coli* strain K.¹² A lack of mutagenic effects was also reported in cultured Chinese hamster lung fibroblasts, using the azaguanine test system,¹³ and in the Chinese hamster ovary cell SCE assay.¹⁴

The results obtained with *Drosophila* were contradictory. No mutagenic effect was noted on mature sex cells of male *Drosophila*.¹⁵ On the other hand, a slight increase in sex-linked recessive lethals was observed following long-term exposure to low concentrations.¹⁶ Because of these positive results, together with the epidemiological studies—in contrast to the various *in vitro* tests—one cannot immediately presume a lack of mutagenicity in mammals. Therefore, halothane was examined in the present study using different test systems in mammals *in vivo*.

One of these tests was the analysis for structural chromosome aberrations in bone marrow cells of Chinese hamsters. The results clearly show that there is no significant difference in the number of structural aberrations between exposed and unexposed animals. In addition to this cytogenetic test, the BrdU-tablet method⁴ was performed to determine *in vivo* induced SCEs. The SCE test is a mutagenicity test system which is presently undergoing validation studies. Although

TABLE 4. Induction of Dominant Lethal Mutations in Mice Exposed to Halothane

Series	Females with Implants	Total Implants		Dead Implants		Living Implants		Dominant Lethal Mutations
		n	Per %	n	Per %	n	Per %	
Controls	30	372	12.40	52	1.73	320	10.67	—
Females exposed to 1 per cent halothane for 5 hours	30	360	12.00	42	1.40	318	10.60	0.66
Males exposed to 1 per cent halothane for 1 hour per day for 48 days	44	527	11.98	56	1.27	471	10.70	-0.28

TABLE 5. Analysis of 16-day-old Living Mice Embryos of Dams Exposed to Halothane During Meiotic Stages

Series	Number of Exposed Dams	Number of Analyzed Embryos	Number of Monosomic Embryos	Number of Trisomic Embryos
Controls	19	200	0	0
1 per cent halothane 1 × 5 hours	21	200	0	0

there are some uncertainties about the exact relation between SCEs and induced structural aberrations,¹⁷ many correlations do exist, and there is no question that SCEs reflect damage to DNA. In this sense the SCE test is an extremely sensitive mammalian mutagenicity test. Using this test, there was no significant increase of SCEs per cell after exposing hamsters to 1 per cent halothane for 3 h, or to 0.5 per cent for 12 h. These negative results support those performed with cell cultures.¹⁴

Despite a lack of chromosome breakage effects, halothane has been reported to induce cellular effects such as delayed cell cycle and segregational errors of chromosomes. In mammalian cells *in vitro*,¹⁸ as well as in 4-day-old chick embryos *in vivo*,¹⁸ the frequency of abnormal telo-anaphases was reported to be increased. These abnormalities included multipolar spindles and chromosome lagging. Similar results were reported¹⁹ using Chinese hamster fibroblasts. Radioautographs of rat epithelial cells, pretreated with ³HT and exposed to halothane, were made by Bruce and Traurig,²⁰ and the percentage of labeled mitotic figures was determined. From these data it was concluded²⁰ that the effect of halothane is one of specifically prolonging the period of DNA synthesis; other phases of the cell cycle apparently were unaffected. The present data, however, are contrary to these conclusions. During the meiotic stages of oogenesis, a replicative DNA synthesis did not occur. Nevertheless, the cell cycle of oocytes of mice exposed during meiotic stages was prolonged (table 3).

The dominant lethal test was performed to investigate whether clinical doses of halothane affect the spindle formation at anaphase I in mammals *in vivo*

and to analyze whether nuclear abnormalities, possibly induced in germ cells, and a prolonged cell cycle increase the preimplantation loss and the number of dead implants. The precise timing for halothane exposure of meiotic stages in non-hormone pretreated females is difficult because of the variation in maturation stages between different mice.²¹ However, the maturation process takes place mainly during the night, and preliminary observations showed that anaphases I in oocytes mainly appear between 10 P.M. and 3 A.M. In the present experiments, female mice were exposed to halothane during this time so that halothane could act at meiotic cell divisions. Immediately thereafter, the females were caged with unexposed males. The results, however, were negative. The number of implants was not significantly decreased. Furthermore, there were no differences in the number of dead and living implants as compared to the controls, and neither monosomic nor trisomic cells occurred in living embryos (tables 4 and 5).

In addition, disturbances like chromosome lagging in mammals *in vivo* should lead to micronuclei. This, however, did not take place. The frequency of micronuclei in erythrocytes of chronically exposed male mice (48 days) was not significantly ($P > 0.02$) increased as compared to controls (table 6).

A further indication that halothane might be mutagenic is the reported increased risk of congenital abnormalities among unexposed wives of male operating room personnel.¹ Therefore, in the present experiments male mice were chronically exposed to halothane. It is known that chemical mutagens can act on all stages of male gametogenesis. To cover the entire gametogenesis, including stem cells, male mice were exposed to halothane for 48 days and mated thereafter with untreated females. Performing the dominant lethal test there was no evidence of mutagenic effects.

From these experiments reported herein, it is not possible to evaluate the possible teratogenic effects of halothane. On the other hand, halothane does not induce mutations (chromosome aberrations, SCEs micronuclei, or dominant lethal mutations) under the conditions tested in mammals *in vivo*.

TABLE 6. Micronucleus Test in Chronically Exposed Male Mice to 1 Per Cent Halothane for 48 Days, 1 h Per Day

Series	Number of Animals	Number of Analyzed Erythrocytes		Micronucleated Polychromatic Erythrocytes		Micronucleated Normochromatic Erythrocytes	
		Polychromatic	Normochromatic	Number	Per Cent ± SD	Number	Per Cent ± SD
Controls	10	10,000	10,000	26	0.26 ± 0.18	19	0.19 ± 0.14
Halothane	10	10,000	10,000	37*	0.37 ± 0.14	35*	0.35 ± 0.17

* $P > 0.02$.

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