

Carcinogen Bioassay of Nitrous Oxide in Mice

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A carcinogen bioassay of nitrous oxide (N₂O) was performed in groups of male and female Swiss-Webster mice exposed to either air (n = 179), 10% N₂O (n = 152), or 40% N₂O (n = 151) for 4 h per day, 5 days per week. After 78 weeks of exposure, there was a 5-week period without treatment following which surviving mice were killed. Mice killed at this time or dying *in extremis* at other times were subjected to complete autopsy unless advanced autolysis or cannibalism precluded examination. Mean body weights for male and female mice in the 10% N₂O group were the same as those in the air control group throughout the study, whereas they were 5% less in the 40% N₂O group. Mean organ weights for N₂O-treated mice were not statistically different from those of control mice. Gross and microscopic examination of tissues revealed a variety of neoplastic and nonneoplastic lesions; however, their presence was unrelated to treatment. (Key words: Anesthetics, gaseous: nitrous oxide. Toxicity: carcinogenicity.)

NITROUS OXIDE (N₂O), in continuous clinical use for more than a century, is still the most commonly used inhaled anesthetic in the United States. Initially, N₂O was considered an inert and nontoxic gas, but recent evidence has definitively linked it to several toxic effects, including hematologic and neurologic dysfunction.¹ In addition, it has putatively been linked to other toxic effects, including carcinogenicity. In a study of dentists and their chairside assistants, a 1.5-fold increase in the general cancer incidence ($P = 0.06$) was noted in women exposed to waste N₂O compared with unexposed women.² The incidence of cancer of the cervix was about 2.5-fold higher than expected ($P = 0.04$). Despite these results, the association between human cancer and N₂O is very tenuous because of the intrinsic weaknesses of such retrospective surveys of cancer incidence, the generally negative results from surveys of cancer death rates, and the fact that interview-

reported findings were not verified by examination of medical records. Thus, to better define the carcinogenic potential of N₂O, we undertook a lifetime carcinogen bioassay in mice using a standard animal carcinogenicity protocol.

Materials and Methods

Two hundred and fifty male and 250 female 3-week-old Swiss-Webster mice** were kept in quarantine for 2 weeks. They were individually ear tagged and divided into three groups. The numbers in each group after elimination of a few mice that failed to thrive during the first few weeks of the study are shown in table 1. Mice were housed not more than four to a cage in polypropylene plastic cages with stainless steel lids and were bedded on ground corn cob.†† They were fed small animal chow‡‡ and were allowed to drink tap water *ad libitum* except during the daily treatment period when food and water were removed. There was a fixed diurnal cycle of 12 h light and 12 h darkness, with N₂O exposure during the light phase. All mice were inspected daily for disease and were weighed at least every 4 weeks. Group 1 (control group) were exposed to compressed air, Group 2 to 10% N₂O, and Group 3 to 40% N₂O for 4 h per day, 5 days per week. Exposures were performed in air-tight, 1000 l capacity, Plexiglas® chambers operating at a negative pressure of 1 cmH₂O. Groups were exposed at the same time each day in three separate chambers. Exposure to 40% N₂O was established in preliminary, subchronic studies as a regimen that was likely to provide the maximum tolerated dose (MTD) of N₂O in the lifetime study, *i.e.*, a dose sufficiently high that it was likely to cause close to but not greater than 10% loss of body weight but unlikely to cause early death.

Animal cages were randomly placed in the chamber. Medical grade N₂O in oxygen and compressed air was delivered to the chambers through rubber tubing. Initial flows were of the order of 100 l/min until the chambers were charged, at which time maintenance flows of 10 l/min were used. Uniform N₂O concentration was maintained by a high-volume recirculation fan and was continuously monitored with a Miran 1A-IF® infrared gas analyzer. Tank standards of N₂O were used to calibrate the gas analyzer before, during, and at the end of each

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TABLE 1. Number of Mice in the Carcinogen Bioassay Study

Treatment	Sex	Start	Unscheduled Deaths	Scheduled Killing (88 weeks)
Air (control)	Male	91	23	68
	Female	88	20	68
10% N ₂ O	Male	75	18	57
	Female	77	14	63
40% N ₂ O	Male	76	36	40
	Female	75	18	57

exposure. Chamber temperature was maintained at $25 \pm 2^\circ \text{C}$, humidity at $50 \pm 10\%$, oxygen at 21–23%, and carbon dioxide concentration at less than 0.3%. Air flow and chamber conditions were kept the same for the three chambers. After 78 weeks of exposure, a 5-week period without treatment was allowed to insure that examination for carcinogenicity was not confounded by acute toxic changes. The period was not extended past 5 weeks because natural attrition of mice would have reduced the size of some groups below that needed for adequate statistical comparisons. Following this period, surviving animals were killed by carbon dioxide overdose.

All animals killed at the scheduled time or dying *in extremis* at other times were subjected to complete autopsy examinations. The only exceptions were eight mice in which cannibalism or advance autolysis precluded examination. The procedures used for the gross autopsy were the same as those recommended by the National Cancer Institute^{§§} and have previously been described in detail.³

More than 40 tissues were examined *in situ*, then dissected from the carcass, incised, and reexamined before being fixed in 10% neutral buffered formalin. Liver, spleen, kidneys, and testes were weighed fresh. After fixation, tissues were again examined grossly, and 32 routine

§§ Sontag JM, Page NT, Saffioti U: Guidelines for carcinogen bioassay in small rodents. NCI-CG-TR-1, Technical Report Series No. 1 DHEW publication no. (NIH) 76-801, 1976.

TABLE 2. Mean \pm SD of Selected Organ Weights (g)*

Organ	Control		10% N ₂ O		40% N ₂ O	
	M	F	M	F	M	F
Liver	2.53 \pm 0.49	1.84 \pm 0.37	2.43 \pm 0.40	1.70 \pm 0.29	2.60 \pm 0.64	1.64 \pm 0.18
Spleen	0.16 \pm 0.09	0.19 \pm 0.17	0.16 \pm 0.11	0.16 \pm 0.13	0.17 \pm 0.15	0.15 \pm 0.09
Right kidney	0.47 \pm 0.10	0.29 \pm 0.06	0.47 \pm 0.08	0.29 \pm 0.05	0.46 \pm 0.07	0.29 \pm 0.04
Left kidney	0.46 \pm 0.10	0.29 \pm 0.05	0.47 \pm 0.09	0.29 \pm 0.05	0.46 \pm 0.07	0.30 \pm 0.04
Right testis	0.08 \pm 0.02		0.08 \pm 0.02		0.08 \pm 0.02	
Left testis	0.09 \pm 0.02		0.08 \pm 0.02		0.07 \pm 0.02	

* Intergroup comparison performed by analysis of variance.

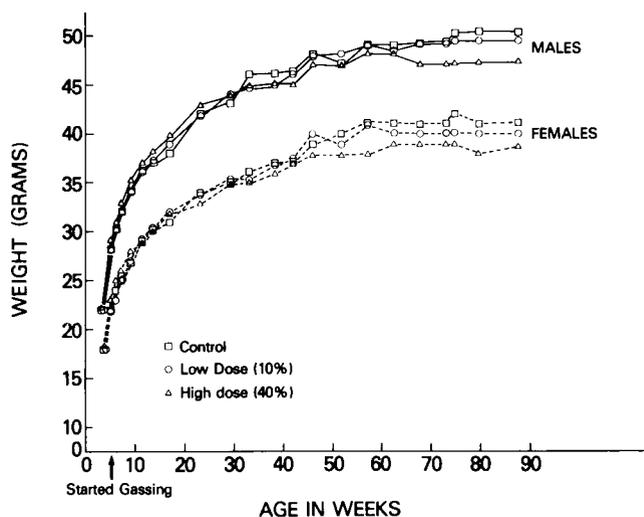


FIG. 1. Mean body weights for male and female mice throughout the study. Mice exposed to 40% N₂O consistently weighed about 5% less than those exposed only to air.

sections from different organs and all abnormal tissues were processed for microscopic examination. Included in this group were bone marrow and blood smear (the latter for red blood cell, differential white blood cell, reticulocyte, and platelet counts). Tissue sections were cut at 4 to 6 μm and stained with hematoxylin and eosin. Microscopic examinations were performed by a pathologist experienced in mouse histology. Examiners were unaware of the treatment groups of the mice at the time of all examinations. Consultation was sought from other pathologists when a histopathologic diagnosis was in doubt.

Intergroup comparisons were made using analysis of variance with post hoc tests, probit analysis, and Chi-square test as appropriate; $P < 0.05$ was considered statistically significant.

Results

Mean body weights for males and females in Group 2 (10% N₂O) were similar to those in Group 1 (control) throughout the study, whereas they were about 5% less

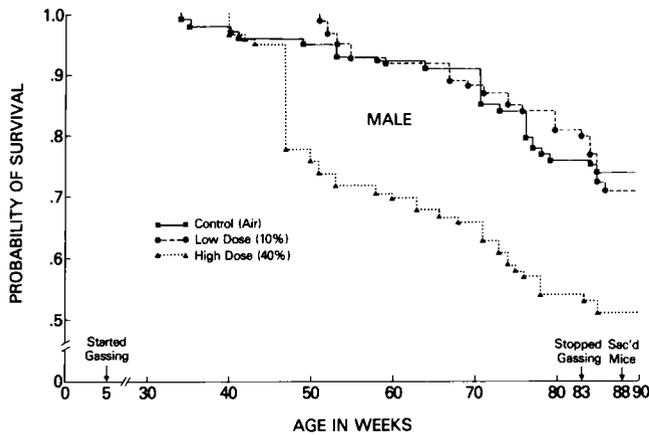


FIG. 2. Survival rates for male mice. The survival rates for mice exposed to air was not statistically different from those exposed to 10% N₂O (Group 2) throughout the study. The percentage of mice surviving to the time of scheduled sacrifice in the 40% N₂O Group (3) was less than that of mice in the control group (1) because of the accidental loss of 12 mice during week 42 of gassing. Otherwise, the survival rates in Group 3 were the same as in the other groups.

for Group 3 (40% N₂O) after about week 60 of gassing for males and week 40 for females (fig. 1). No other gross toxic effects of treatment were noted. Mean organ weights of N₂O-treated mice were not statistically different from those of control mice (table 2) nor were mean organ weights expressed as a percentage of body weight different from controls.

The percentages of male and female mice surviving the study are shown in figures 2 and 3, respectively. On one day during week 42 of gassing, an event occurred that led to the death of 12 male and 2 female mice exposed to 40% N₂O. There was a failure of the air conditioner in the building in which the exposure chambers were kept. The result was an undue rise in temperature during the last 2 h of gassing, especially in the 40% N₂O chamber.

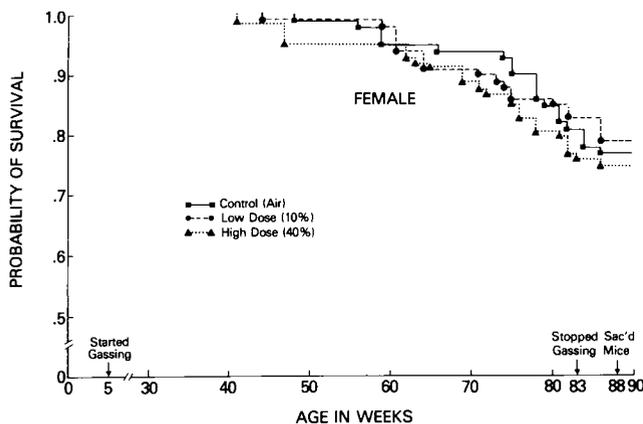


FIG. 3. Survival rates for female mice. The survival rates were not statistically different among the groups throughout the study.

TABLE 3. Percentage of Mice with Tumors Observed by Gross Examination*

Tumors	Control		10% N ₂ O		40% N ₂ O	
	M	F	M	F	M	F
Lung adenomas	37.4	28.4	37.3	31.2	21.1	41.3
Liver tumors	12.1	4.5	10.6	2.6	7.6	2.7
Others	9.9	21.6	13.3	14.3	10.5	14.6

* Intergroup comparisons performed by chi-square test.

Mice that survived this event showed no long-term sequelae. The reason that more males died than females is unknown although, in general, males tend to be more debilitated in long-term studies because of infection from fighting. When mice that died from hyperpyrexia were excluded from the probit analysis, the survival rates for all groups throughout the study were the same.

Gross and microscopic examination of tissues revealed a variety of nonneoplastic lesions including ovarian cysts, cholecystitis, bladder stones, and testicular atrophy. Their presence was unrelated to treatment. In general, there was no microscopic evidence of cellular damage and, in particular, a detailed examination of blood smears and bone marrow sections showed no evidence of megaloblastic changes or bone marrow depression.

The first neoplastic lesion appeared 26 weeks after the start of treatment, and numerous other tumors appeared throughout the study (table 3). As in similar studies with other anesthetics,³⁻⁶ most of the tumors in both control and test animals were either lung adenomas of alveolar cell origin or liver tumors of the basophilic hepatocellular adenoma type. There were no statistical differences among the groups either in the total number of tumors or in the number of tumors of a particular cell type. It should be noted, however, that the background incidence of lung adenomas in this study was high and, thus, the statistical power for the analysis of differences among the groups for this tumor type was low.

Discussion

Nitrous oxide is the most widely used inhaled anesthetic. In the United States alone, it is estimated that 20 million surgical and 4.5 million dental patients receive N₂O each year.¹¹ Furthermore, at least 200,000 operating, dental, and veterinary personnel are chronically exposed to trace concentrations of N₂O.¹¹ In addition to its medical use, N₂O is used as a propellant in spray cans

¹¹ US Department of Health, Education and Welfare: NIOSH criteria for a recommended standard: Occupational exposure to waste anesthetic gases and vapors. DHEW publication no. (NIOSH) 77-140. Public Health Service, Center for Disease Control, (NIOSH), March 1977.

and aerosols and for several other commercial and industrial purposes. It is also the most abundant atmospheric nitrogen compound, with a mean concentration of about 0.25 ppm in clean and 1.0 ppm in polluted air.⁷ Thus, if N₂O has carcinogenic or other toxic potential, its widespread use and distribution would make it a considerable public health hazard.

In the present study, chronic administration of high concentrations of N₂O had no morphologic or functional effects on the mouse hemopoietic system. This is surprising because N₂O is known to inactivate vitamin B₁₂ very rapidly in rodents and to produce a number of biochemical derangements, including interference with normal methionine and thymidine synthesis.⁸ Nevertheless, the results agree with our previous study in which no adverse hemopoietic effects were observed after 14 weeks of exposure of Swiss-Webster mice to 50% N₂O for 4 h per day, 5 days per week.⁹ Other species, such as bats,¹⁰ monkeys,¹¹ and humans,¹² not only show the biochemical effects of inactivation of vitamin B₁₂ but also develop megaloblastic anemia and subacute combined degeneration of the cord. The reasons for the different functional effects of N₂O in several species are unknown.

Although there are species differences in some types of toxicity, numerous studies over the last two decades have indicated that results of lifetime studies in small rodents predict the carcinogenic potential of a drug in humans.¹³ For example, aflatoxin B₁, vinyl chloride, benzidine, and diethylstilbestrol have about the same carcinogenic potential in at least one species of rodent as in humans. Thus, the lack of carcinogenicity of N₂O in our study is encouraging, especially considering our protocol involved lifetime exposure, which is needed to test adequately the carcinogenic potential of any drug.¹⁴

Two previous animal studies have been performed to determine whether N₂O is a carcinogen. Eger *et al.*⁵ exposed Swiss-ICR mice to 12.5% or 50% N₂O for 2-h periods both *in utero* during the last half of pregnancy (four exposures) and after delivery (24 exposures at 2- to 3-day intervals). They found no evidence of carcinogenic activity. However, as previously discussed,⁴ two aspects of this study lead to a low confidence in the significance of the negative results. First, the total dosage of N₂O was very low and, second, mice were exposed only at the beginning of their lives as opposed to the accepted protocol of exposure for at least 18 months. In the second study, Coate *et al.*⁶ exposed groups of Fischer-344 rats either to 1 ppm

halothane and 50 ppm N₂O, or to 10 ppm halothane and 500 ppm N₂O for 7 h per day, 5 days per week for 104 weeks; no carcinogenic activity was seen. Because the number of rats and the dosages used were small, the statistical power of this study is low. Nevertheless, these studies, together with our study in which N₂O was administered for 18 months at the MTD suggest that N₂O has little or no carcinogenic potential.

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