

Reproductive and Teratogenic Effects of Nitrous Oxide, Isoflurane, and Their Combination in Sprague-Dawley Rats

Masahiko Fujinaga, M.D.,* Jeffrey M. Baden, M.D.,† Edgar O. Yhap, M.D.,‡ Richard I. Mazze, M.D.§

The reproductive and teratogenic effects of nitrous oxide (N_2O), isoflurane, and their combination were studied in 130 timed-pregnant rats. Rats were exposed to either air, 0.35% isoflurane (1/4 MAC), 50% N_2O (a known teratogenic concentration), or 50% N_2O plus 0.35% isoflurane for 24 h on day 8 of pregnancy. On day 20 of pregnancy, cesarean sections were performed; a total of 1268 offspring were delivered and immediately examined for external abnormalities. They were subsequently examined microscopically either for visceral or skeletal abnormalities. N_2O caused significantly higher incidences of early and late resorptions, and major visceral malformations. The addition of isoflurane to N_2O prevented the majority of these adverse effects. These results cast doubt on the methionine synthase inhibition theory of N_2O teratogenicity. (Key words: Anesthetics, gases; nitrous oxide. Anesthetics, volatile; isoflurane. Pregnancy; teratogenicity. Toxicity; fetal, teratogenicity; reproductive. Toxicity; nitrous oxide; isoflurane.)

FOR THE PAST 10 yr, we have studied the reproductive and teratogenic potential of anesthetic agents using several rodent models.¹⁻¹¹ To date, adverse effects only have been seen consistently with nitrous oxide (N_2O),^{5,6,8,11} confirming the original report in 1967 by Fink *et al.*¹² and the "rediscovery" of this phenomenon in 1980 by Lane *et al.*¹³ In clinical practice, N_2O is seldom administered alone; rather, it is usually combined with other inhaled or intravenous anesthetics. Thus, the question arises whether the combination of N_2O administered with other agents has a different reproductive effect than that of N_2O administered alone. In a previous study, we gave 35% or 50% N_2O alone and in combination with fentanyl, 500 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$.¹¹ The addition of the narcotic did not significantly add to the adverse reproductive and teratogenic effects of N_2O alone. Since isoflurane is the most commonly used potent inhalational anesthetic in

the United States today, we studied its reproductive effects in combination with N_2O .

Methods

A total of 130, 9-11-week-old, timed-pregnant Sprague-Dawley rats were obtained from the breeder[¶] on day 6 of pregnancy, and labelled individually with metal ear tags (day 0 was defined as the day when a copulatory plug was found in the vagina). Rats were bedded on ground corncob,^{**} housed four per cage and fed standard laboratory rodent food^{††} and tap water *ad libitum*. Temperature in the animal room was maintained at 21-24° C, and artificial light was provided from 6 am to 7 pm each day. Upon receipt, rats were weighed then randomly divided into four groups of approximately equal average weight, as follows: 1) control group (n = 40); 2) N_2O group (50% N_2O , n = 30); 3) isoflurane group (0.35% isoflurane, n = 30); and 4) N_2O plus isoflurane group (50% N_2O plus 0.35% isoflurane, n = 30).

On day 8 of pregnancy, starting at 9 am, rats were administered either air, 0.35% isoflurane, 50% N_2O , or 50% N_2O plus 0.35% isoflurane for 24 h without food and water. Fifty percent N_2O administered for 24 h on day 8 is a proven teratogenic dose.¹¹ Isoflurane, 0.35%, which is 1/4 MAC in rats,¹⁴ is the highest concentration which can be administered to Sprague-Dawley rats in combination with 50% N_2O without causing death.^{‡‡} Exposures to the anesthetic agents were performed simultaneously in gas-tight, Plexiglas[®] chambers of approximately 1,000-l capacity. Medical grade N_2O and oxygen were delivered to the chambers at a total flow of 20-30 $\text{l} \cdot \text{min}^{-1}$, and were mixed with room air to achieve the desired N_2O and oxygen concentrations. N_2O concentrations were monitored continuously with Miran 1A-1F[®] infrared analyzers and were recorded on strip-chart recorders; they were maintained within 5% of the desired level. Oxygen concentrations were monitored continuously with IL402[®] analyzers and were

* Postdoctoral Research Fellow in Anesthesia (SUSM and PAVAMC).

† Associate Professor (SUSM) and Staff Anesthesiologist (PAVAMC).

‡ Associate Professor (Clinical) (SUSM) and Staff Anesthesiologist (PAVAMC).

§ Professor (SUSM) and Chief (PAVAMC).

Received from the Department of Anesthesia, Stanford University School of Medicine (SUSM), Stanford, California; and Anesthesiology Service, Palo Alto Veterans Administration Medical Center (PAVAMC), Palo Alto, California. Accepted for publication July 31, 1987. Supported by the Veterans Administration and the Anesthesia/Pharmacology Research Foundation.

Address reprint requests to Dr. Mazze: Anesthesiology Service 112A, Palo Alto V.A. Medical Center, 3801 Miranda Avenue, Palo Alto, California 94304.

¶ Hilltop Lab Animals, Inc., Scottsdale, PA 15683.

** Bed-O'Cobs, Anderson's Cob Division, Maumee, OH 43537.

†† Wayne Lab Blox, Allied Mills, Inc., Chicago, IL 60606.

‡‡ In preliminary studies, 1.1% isoflurane plus 50% N_2O resulted in 100% mortality secondary to hypothermia after 6 h (n = 6); 0.7% isoflurane plus 50% N_2O resulted in 75% mortality in 10 h (n = 12); and 0.35% isoflurane plus 50% N_2O caused no deaths after 24 h (n = 24).

TABLE 1. Body Weights and Body Weight Changes, Grams (Mean \pm SD)

	Control	0.35% ISO	50% N ₂ O	0.35% ISO + 50% N ₂ O
No. of rats studied	40	30	30	30
No. of rats pregnant	37	27	26	26†
Pregnancy rate (%)	93	90	87	87
Weight				
Day 6 of pregnancy‡ (On arrival)	211 \pm 15	209 \pm 11	210 \pm 12	210 \pm 11
Day 8 of pregnancy (before exposure)	233 \pm 15	225 \pm 16	231 \pm 13	231 \pm 12
Day 9 of pregnancy (after exposure)	212 \pm 15	207 \pm 14	208 \pm 11	206 \pm 11
Day 12 of pregnancy	254 \pm 17	231 \pm 33*	241 \pm 12*	237 \pm 13*
Day 14 of pregnancy	271 \pm 18	245 \pm 44*	259 \pm 14	256 \pm 16*
Day 16 of pregnancy	290 \pm 21	272 \pm 38*	279 \pm 24	275 \pm 28*
Day 20 of pregnancy (at Cesarean section)	348 \pm 32	337 \pm 37	328 \pm 24*	328 \pm 28*
Weight loss during the exposure	21 \pm 4	18 \pm 3	24 \pm 5	26 \pm 3*

ISO = isoflurane.

* $P < 0.05$ vs. control.

† One litter had no live fetuses.

‡ The day a copulatory plug was observed in the vagina was defined as day 0 of pregnancy.

maintained at 22–25%. Isoflurane was vaporized in a bubble-through vaporizer using medical grade compressed air as the carrier gas and was delivered to the chambers through latex rubber tubing. Isoflurane concentrations were monitored continuously with Miran 1A-1F[®] infrared analyzers and were recorded on strip-chart recorders; they also were maintained within 5% of the desired level. Rats in the control group were exposed to room air. Temperature in the chambers ranged from 20–30° C, averaging 24° C. Carbon dioxide concentrations were not measured; however, in previous experiments employing similar conditions, it has ranged from 0.1–0.2%.⁶ All rats were weighed before and after exposure and every 2–4 days during the experiment.

On day 20 of pregnancy, rats were killed by carbon dioxide inhalation, and cesarean sections were performed. The uterus was examined, and the number and position of live and dead fetuses, resorptions, and implantations were recorded. The weight and sex of each live fetus were determined, and each fetus was examined for evidence of external abnormalities. Every other fetus was fixed in 70% ethanol and macerated with potassium hydroxide. The skeleton was then stained with alizarin red S using the modified method of Staples and Schnell,¹⁵ cleared with glycerol, and subsequently examined microscopically for skeletal abnormalities. The remainder of the fetuses were preserved in Bouin's solution and subsequently dissected and examined microscopically for visceral abnormalities, as described by Barrow and Taylor.¹⁶ All examinations were done without knowledge of the treatment groups.

An early resorption was considered present when an implantation site was defined but fetal parts could not be identified. A late resorption had identifiable fetal parts. Abnormalities were classified as follows. Fetal morphologic abnormalities that altered general body conformity, would have disrupted or interfered with

bodily functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomic structure that would have no significant biological effect on the rats' health or on their body conformity, and represented only slight deviations from normal, were categorized as developmental variants. Abnormalities which did not fall under the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies. Fetuses weighing 25% less than the mean weight of their litter were classified as runts.

STATISTICAL ANALYSES

The percentage of fetuses affected in each litter was computed for each type of abnormality. Data were analyzed by one-way analysis of variance (ANOVA). Student's *t* test, corrected for multiple analyses (Bonferroni), was used as an *a posteriori* test when differences were found with ANOVA. $P < 0.05$ was considered significant.

Results

MATERNAL EFFECTS

No rats died prior to cesarean section on day 20 of pregnancy. Rats exposed to 0.35% isoflurane or to 50% N₂O appeared mildly sedated and rested quietly during the exposure; they occasionally changed their posture and moved about the cage. Rats exposed to the combined treatment appeared more sedated and changed position less frequently than those in the other groups. Compared with the control group, significant weight loss occurred during the 24-h exposure only in the combined treatment group. However, significant weight loss occurred on day 12 in all three experimental groups, and at other times in the experiment as indicated in table 1.

TABLE 2. Maternal and Fetal Observations at Cesarean Section (Mean \pm SD)

	Control	0.35% ISO	50% N ₂ O	0.35% ISO + 50% N ₂ O
No. of rats examined	37	27	26	26†
Total implantations/rat	12.2 \pm 3.3	12.7 \pm 2.0	12.2 \pm 2.3	12.0 \pm 2.6
Total live fetuses/rat	11.7 \pm 3.4	11.8 \pm 2.8	9.5 \pm 3.5	10.6 \pm 3.2
Early resorptions/rat (%)	4.9 \pm 10.3	7.4 \pm 14.8	18.0 \pm 19.9*	12.4 \pm 20.0
Late resorptions/rat (%)	0.0 \pm 0.0	0.0 \pm 0.0	6.8 \pm 11.7*	0.6 \pm 2.2
Dead in utero/rat (%)	0.0 \pm 0.0	0.0 \pm 0.0	1.0 \pm 2.9	0.6 \pm 2.2
Total fetal wastage/rat (%)	4.9 \pm 10.3	7.4 \pm 14.8	25.9 \pm 28.0*	13.6 \pm 20.9
Mean fetal body weight (g)	4.4 \pm 0.5	4.4 \pm 0.6	4.4 \pm 0.7	4.4 \pm 0.6
Female fetuses (%) /rat	46.9 \pm 19.3	53.8 \pm 14.5	48.8 \pm 19.4	51.8 \pm 18.0

ISO = isoflurane.

* $P < 0.05$ vs. control.

† One litter had no live fetuses.

REPRODUCTIVE EFFECTS

There were no significant differences among the four groups in pregnancy rate (table 1), total number of implantations and live fetuses per rat, and sex ratio (table 2). Mean fetal weight was same among all the groups. The incidences of early resorptions, late resorptions, and total fetal wastage were significantly higher than control only in the 50% N₂O group. The incidences of all categories of fetal wastage in the combined treatment group were between those of the control group and the 50% N₂O group.

TERATOGENIC EFFECTS

A total of 1268 offspring were delivered, and all were examined for external abnormalities (table 3). Subsequently, 632 fetuses were examined for visceral and 636 for skeletal abnormalities. There were no signifi-

cant differences among the groups in the external examinations. The incidences of major visceral malformations and of any visceral abnormality were significantly higher only in the 50% N₂O group. The predominant lesion was a right-sided aortic arch, which was present in fetuses from 5 of the 26 litters exposed to 50% N₂O. The incidences of skeletal developmental variants and of any skeletal abnormality (which incorporates developmental variants) were significantly higher in both the 50% N₂O and combined treatment groups. However, there were no differences in the incidences of major or minor skeletal abnormalities among all of the groups.

Discussion

The reproductive and teratogenic effects of N₂O in a mammalian model were first reported by Fink *et al.*¹² in 1967. They exposed pregnant rats to 45–50% N₂O for 2, 4, or 6 days starting on day 8 of pregnancy, and

TABLE 3. Results of Fetal Examinations (Mean Percent Abnormal Fetuses Per Rat \pm SD)

	Control	0.35% ISO	50% N ₂ O	0.35% ISO + 50% N ₂ O
No. of rats examined	37	27	26†	25
External examinations				
No. of fetuses examined	433	319	241	275
Any external abnormalities (%)	0.2 \pm 1.2	0.4 \pm 1.9	0.7 \pm 3.3	0.3 \pm 1.6
Major malformations (%)	0.2 \pm 1.2	0.0 \pm 0.0	0.7 \pm 3.3	0.0 \pm 0.0
Minor anomalies (%)	0.2 \pm 1.2	0.4 \pm 1.9	0.0 \pm 0.0	0.3 \pm 1.6
Runt (%)	1.5 \pm 3.5	1.0 \pm 4.1	1.6 \pm 4.7	0.9 \pm 2.6
Visceral examinations				
No. of fetuses examined	216	157	123	136
Any visceral abnormalities (%)	10.9 \pm 12.3	10.0 \pm 20.3	26.4 \pm 32.5*	13.4 \pm 21.1
Major malformations (%)	0.0 \pm 2.1	0.0 \pm 0.0	14.9 \pm 30.2*	4.7 \pm 14.1
Minor anomalies (%)	10.0 \pm 10.3	10.0 \pm 20.3	17.1 \pm 19.3	10.7 \pm 18.7
Skeletal examinations				
No. of fetuses examined	217	162	118	139
Any skeletal abnormalities (%)	16.4 \pm 21.0	24.5 \pm 25.2	34.7 \pm 29.7*	35.2 \pm 28.2*
Major malformations (%)	0.4 \pm 2.1	0.0 \pm 0.0	0.0 \pm 0.0	0.7 \pm 3.4
Minor anomalies (%)	0.8 \pm 3.6	3.1 \pm 11.3	5.5 \pm 12.4	0.6 \pm 2.8
Developmental variants (%)	15.2 \pm 20.8	23.3 \pm 23.8	33.0 \pm 28.0*	34.5 \pm 28.8*

ISO = isoflurane.

* $P < 0.05$ vs. control.

† One litter had only one live fetus and it was examined for visceral abnormalities.

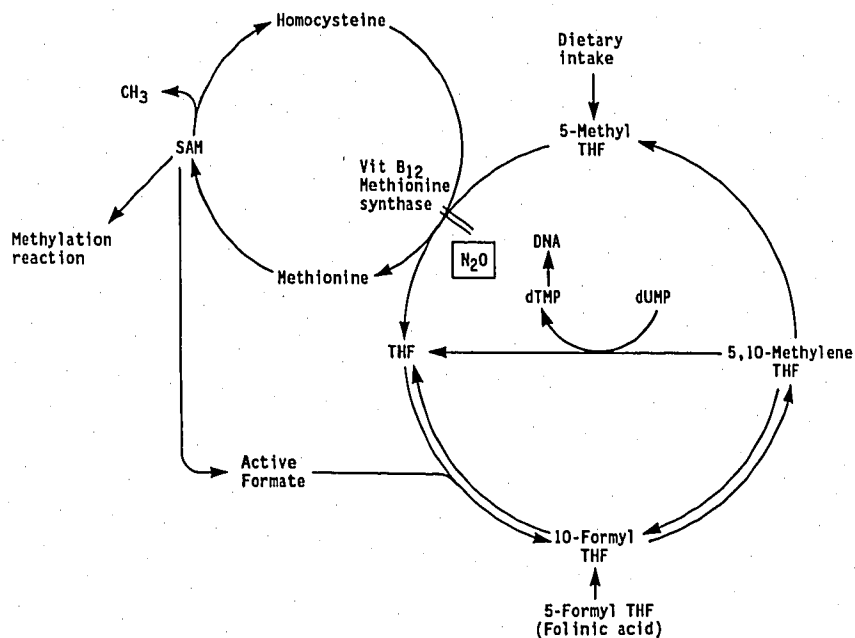


FIG. 1. Pathway depicting the inhibition of methionine synthase by N_2O and the consequences of interference with this reaction. THF = tetrahydrofolate; SAM = s-adenosyl methionine; Vit B₁₂ = vitamin B₁₂; dTMP = thymidine; dUMP = deoxyuridine.

observed higher incidences of fetal resorptions and deaths, runts, and vertebral and rib abnormalities. In 1980, Lane *et al.*¹³ confirmed these findings. They exposed pregnant rats to 70–75% N_2O for 24 h on day 8 of pregnancy, and to 70–75% xenon, a slightly more potent anesthetic than N_2O but with no known biochemical effects. Nitrous oxide treatment resulted in increased fetal wastage and in teratogenic effects; xenon did not. They concluded that the teratogenic effects of N_2O were not related to intrinsic anesthetic mechanisms, but were specific to the drug. Our N_2O studies also have confirmed that it is teratogenic in rats, and we have excluded potential confounding factors, such as starvation, shipping dams while pregnant, and restraining them during exposure, as the cause of this effect.⁶ We also have defined the threshold of N_2O teratogenicity in rats: concentrations of 35% or less for 24 h do not produce adverse reproductive effects when administered on day 8 of pregnancy, but concentrations of 50% or more are harmful.^{6,11}

One of the aims of our research has been to test drug combinations similar to those used in clinical practice. Thus, in an earlier study,¹¹ we tested the reproductive toxicity of N_2O combined with fentanyl. We administered 50% N_2O for 24 h on day 8 of pregnancy to rats in which we had implanted osmotic minipumps that delivered $500 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of fentanyl, the maximum tolerated dose of that narcotic. Reproductive toxicity was not increased compared with that of N_2O alone. In the present study, we tested the toxic potential of the combination of 50% N_2O and 0.35% isoflurane (1/4 MAC). All rats survived the combined treatment, al-

though it appeared more stressful than was treatment with N_2O or isoflurane alone, as evidenced by greater weight loss during the 24-h exposure (table 1). Based on the results of our previous study of N_2O combined with fentanyl¹¹ and general toxicological principles, we anticipated that the combination of N_2O and isoflurane either would not increase the reproductive toxicity of N_2O , or would make it worse. To our surprise, we found that the combined treatment group did not differ from control, whereas the N_2O -treated group experienced both adverse reproductive and teratogenic effects. We did not design the present experiment to examine the hypothesis that the treatments differed from one another. To do that would have necessitated a large number of statistical comparisons, thereby increasing the likelihood of a type I error.

The results of our study lead us to doubt the commonly held biochemical theory of N_2O teratogenesis. That theory is based on the original observation by Banks *et al.*,¹⁷ who showed that N_2O oxidized the cobalt of vitamin B₁₂ *in vitro*, converting cob(I)alamin to cob(II)alamin. A similar reaction occurs *in vivo*, resulting in the inactivation of methionine synthase,^{18–20} which requires vitamin B₁₂ in the fully reduced state for normal activity. As a consequence of the inhibition of methionine synthase activity, homocysteine is not converted to methionine, nor is 5-methyltetrahydrofolate demethylated (fig. 1). Decreased availability of methionine and of tetrahydrofolate are presumed to result in decreased levels of 5,10-methylenetetrahydrofolate, which is the one-carbon donor for the conversion of deoxyuridine (dUMP) to deoxythymidine (dTMP); the

latter is one of the four essential DNA bases. By inhibiting methionine synthase activity, N₂O administration interferes with DNA production and, thus, is said to be teratogenic.²¹⁻²³

The effects of general anesthetics on methionine synthase activity have been studied in surgical patients and in animals. In humans, exposure to 50–70% N₂O for 1.3–2.8 h resulted in dose-related decreases in hepatic methionine synthase activity of 25–75%.²⁴ Depression occurs even more rapidly in rats.¹⁸ In mice, N₂O treatment decreases methionine synthase activity, but anesthesia with halothane, enflurane, and isoflurane have no effect.¹⁹ It is not known whether any of these agents prevent the inhibitory effects of N₂O on methionine synthase activity, but that seems unlikely. Thus, for isoflurane to protect against the adverse reproductive effects of N₂O, one has to postulate that it does so by altering another biochemical step. Alternatively, isoflurane may act by preventing a physiologic effect of N₂O, such as the decrease in uterine blood flow that it causes.²⁵ These explanations for the adverse reproductive effects of N₂O in rodents are speculative. However, lack of a definitive explanation for N₂O teratogenicity does not mitigate our findings, which cast doubt on the methionine synthase inhibition theory of N₂O teratogenicity.

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