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

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**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 $\mu g \cdot kg^{-1} \cdot day^{-1}$.[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 $l \cdot min^{-1}$, 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

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‡‡ 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 ± SD)

| | Control | 0.35% ISO | 50% $N_2O$ | 0.35% ISO + 50% $N_2O$ |
|---|---|---|---|---|
| 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 ± 15 | 209 ± 11 | 210 ± 12 | 210 ± 11 |
| Day 8 of pregnancy (before exposure) | 233 ± 15 | 225 ± 16 | 231 ± 13 | 231 ± 12 |
| Day 9 of pregnancy (after exposure) | 212 ± 15 | 207 ± 14 | 208 ± 11 | 206 ± 11 |
| Day 12 of pregnancy | 254 ± 17 | 231 ± 33* | 241 ± 12* | 237 ± 13* |
| Day 14 of pregnancy | 271 ± 18 | 245 ± 44* | 259 ± 14 | 256 ± 16* |
| Day 16 of pregnancy | 290 ± 21 | 272 ± 38* | 279 ± 24 | 275 ± 28* |
| Day 20 of pregnancy (at Cesarean section) | 348 ± 32 | 337 ± 37 | 328 ± 24* | 328 ± 28* |
| Weight loss during the exposure | 21 ± 4 | 18 ± 3 | 24 ± 5 | 26 ± 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% $N_2O$ 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 ± SD)

| | Control | 0.35% ISO | 50% $N_2O$ | 0.35% ISO + 50% $N_2O$ |
|---|---|---|---|---|
| No. of rats examined | 37 | 27 | 26 | 26† |
| Total implantations/rat | 12.2 ± 3.3 | 12.7 ± 2.0 | 12.2 ± 2.3 | 12.0 ± 2.6 |
| Total live fetuses/rat | 11.7 ± 3.4 | 11.8 ± 2.8 | 9.5 ± 3.5 | 10.6 ± 3.2 |
| Early resorptions/rat (%) | 4.9 ± 10.3 | 7.4 ± 14.8 | 18.0 ± 19.9* | 12.4 ± 20.0 |
| Late resorptions/rat (%) | 0.0 ± 0.0 | 0.0 ± 0.0 | 6.8 ± 11.7* | 0.6 ± 2.2 |
| Dead in utero/rat (%) | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.0 ± 2.9 | 0.6 ± 2.2 |
| Total fetal wastage/rat (%) | 4.9 ± 10.3 | 7.4 ± 14.8 | 25.9 ± 28.0* | 13.6 ± 20.9 |
| Mean fetal body weight (g) | 4.4 ± 0.5 | 4.4 ± 0.6 | 4.4 ± 0.7 | 4.4 ± 0.6 |
| Female fetuses (%)/rat | 46.9 ± 19.3 | 53.8 ± 14.5 | 48.8 ± 19.4 | 51.8 ± 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% $N_2O$ group. The incidences of all categories of fetal wastage in the combined treatment group were between those of the control group and the 50% $N_2O$ 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 significant 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% $N_2O$ group. The predominant lesion was a right-sided aortic arch, which was present in fetuses from 5 of the 26 litters exposed to 50% $N_2O$. The incidences of skeletal developmental variants and of any skeletal abnormality (which incorporates developmental variants) were significantly higher in both the 50% $N_2O$ 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 $N_2O$ in a mammalian model were first reported by Fink *et al.*[12] in 1967. They exposed pregnant rats to 45–50% $N_2O$ 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 ± SD)

| | Control | 0.35% ISO | 50% $N_2O$ | 0.35% ISO + 50% $N_2O$ |
|---|---|---|---|---|
| No. of rats examined | 37 | 27 | 26† | 25 |
| External examinations | | | | |
| No. of fetuses examined | 433 | 319 | 241 | 275 |
| Any external abnormalities (%) | 0.2 ± 1.2 | 0.4 ± 1.9 | 0.7 ± 3.3 | 0.3 ± 1.6 |
| Major malformations (%) | 0.2 ± 1.2 | 0.0 ± 0.0 | 0.7 ± 3.3 | 0.0 ± 0.0 |
| Minor anomalies (%) | 0.2 ± 1.2 | 0.4 ± 1.9 | 0.0 ± 0.0 | 0.3 ± 1.6 |
| Runt (%) | 1.5 ± 3.5 | 1.0 ± 4.1 | 1.6 ± 4.7 | 0.9 ± 2.6 |
| Visceral examinations | | | | |
| No. of fetuses examined | 216 | 157 | 123 | 136 |
| Any visceral abnormalities (%) | 10.9 ± 12.3 | 10.0 ± 20.3 | 26.4 ± 32.5* | 13.4 ± 21.1 |
| Major malformations (%) | 0.0 ± 2.1 | 0.0 ± 0.0 | 14.9 ± 30.2* | 4.7 ± 14.1 |
| Minor anomalies (%) | 10.0 ± 10.3 | 10.0 ± 20.3 | 17.1 ± 19.3 | 10.7 ± 18.7 |
| Skeletal examinations | | | | |
| No. of fetuses examined | 217 | 162 | 118 | 139 |
| Any skeletal abnormalities (%) | 16.4 ± 21.0 | 24.5 ± 25.2 | 34.7 ± 29.7* | 35.2 ± 28.2* |
| Major malformations (%) | 0.4 ± 2.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.7 ± 3.4 |
| Minor anomalies (%) | 0.8 ± 3.6 | 3.1 ± 11.3 | 5.5 ± 12.4 | 0.6 ± 2.8 |
| Developmental variants (%) | 15.2 ± 20.8 | 23.3 ± 23.8 | 33.0 ± 28.0* | 34.5 ± 28.8* |

ISO = isoflurane.
* $P < 0.05$ *vs.* control.
† One litter had only one live fetus and it was examined for visceral abnormalities.

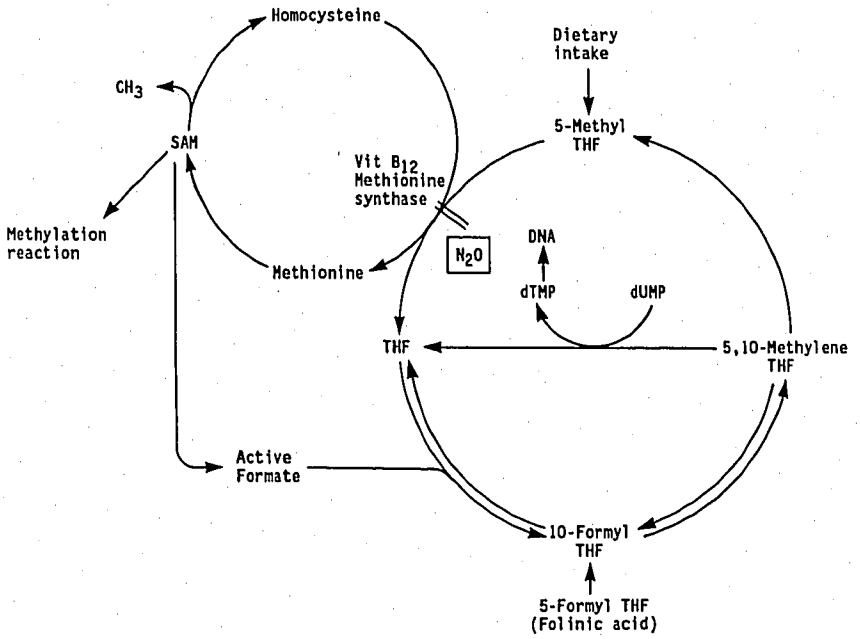


FIG. 1. Pathway depicting the inhibition of methionine synthase by $N_2O$ and the consequences of interference with this reaction. THF = tetrahydrofolate; SAM = s-adenosyl methionine; Vit $B_{12}$ = vitamin $B_{12}$; dTMP = thymidine; dUMP = deoxyuridine.

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 $\mu g \cdot kg^{-1} \cdot day^{-1}$ 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, although 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 fentanyl[11] 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 $B_{12}$ *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 $B_{12}$ 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, $N_2O$ 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% $N_2O$ 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, $N_2O$ 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 $N_2O$ on methionine synthase activity, but that seems unlikely. Thus, for isoflurane to protect against the adverse reproductive effects of $N_2O$, one has to postulate that it does so by altering another biochemical step. Alternatively, isoflurane may act by preventing a physiologic effect of $N_2O$, such as the decrease in uterine blood flow that it causes.[25] These explanations for the adverse reproductive effects of $N_2O$ in rodents are speculative. However, lack of a definitive explanation for $N_2O$ teratogenicity does not mitigate our findings, which cast doubt on the methionine synthase inhibition theory of $N_2O$ teratogenicity.

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