Methionine Prevents Nitrous Oxide-induced Teratogenicity in Rat Embryos Grown in Culture

Masahiko Fujinaga, M.D.,* Jeffrey M. Baden, M.D.†

Background: Nitrous oxide (N₂O)–induced teratogenicity in rats is commonly believed to be due to decreased tetrahydrofolate, which results in decreased DNA synthesis. The role of decreased methionine has been largely ignored as have the sympathomimetic effects of N₂O.

Methods: A rat whole-embryo culture system was used to determine whether N₂O-induced teratogenicity can be prevented with supplemental methionine or folinic acid and whether N₂O-induced situs inversus is mediated by α₁-adrenergic stimulation. Embryos were explanted on day 9 of gestation, and those at stage 10 b (late primitive streak stage) were cultured with or without N₂O and the various chemicals, methionine (25 μg · ml⁻¹), folinic acid (5 μg · ml⁻¹), phenylephrine (range 0.5–50 μM) and prazosin (10 μM). Embryos in the N₂O groups were exposed to a concentration of 75% for the first 24 h of culture. After 50 h of culture, embryos were examined for abnormalities including situs inversus.

Results: Treatment with N₂O alone resulted in increased incidences of malformations and growth retardation. Methionine, but not folinic acid or prazosin, almost completely prevented N₂O-induced malformations and growth retardation. N₂O itself did not cause situs inversus but increased the incidence of phenylephrine-induced situs inversus. This additive effect was blocked by prazosin.

Conclusions: Our results indicate that decreased methionine rather than decreased tetrahydrofolate plays the major role in N₂O-induced teratogenicity in rats. They also indicate that N₂O stimulates the α₁-adrenergic pathway in the embryo and thereby increases the incidence of phenylephrine-induced situs inversus. (Key words: Anesthetics, gases: nitrous oxide. Sympathetic nervous system: sympathomimetics. Toxicity: teratogenicity.)

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IN the late 1960s, Fink and Shepard¹,² published their seminal work on the teratogenicity of nitrous oxide (N₂O) in rats. Since then, many investigators have examined this phenomenon, yet, its mechanisms have remained elusive. A possible breakthrough occurred when anesthesiologists became aware that N₂O inhibited the enzyme methionine synthase, thereby decreasing the intracellular synthesis of both tetrahydrofolate and methionine.³ Particular emphasis was placed on the decrease in tetrahydrofolate, which was known to lead to decreased DNA synthesis. However, in vivo studies have failed to show that supplementation with folates protects the offspring of rats from the effects of N₂O.³,⁵ The protective effects of methionine supplementation has not been investigated. More recently, the sympathomimetic effects of N₂O have also received attention as a possible cause of some malformations, especially situs inversus which is known to be caused by α₁-adrenergic agonists.⁶–⁸

We have established a rat whole-embryo culture model to assist in examining the mechanisms of N₂O-induced teratogenicity.⁹–¹¹ This model has several advantages over in vivo experimental models that have been used previously.¹,²,⁴,⁵,¹²–¹⁹ For example, it has enabled us to separate embryonic effects of N₂O from maternal effects and to more precisely control experimental conditions. In the current study, we aimed to use this system to examine preventive effects of methionine and folinic acid against N₂O-induced teratogenicity, and to determine whether N₂O-induced situs inversus is mediated by α₁-adrenergic stimulation.

Materials and Methods

Animals

Sprague-Dawley rats were obtained from the breeder (Bantin & Kingman, Fremont, CA), housed two per cage and provided with food and water on demand. Temperature in the animal room was maintained at 21–24°C and artificial lighting was provided between
6 AM and 6 PM each day. Timed-pregnant rats were obtained by mating the rats for 2 h between 8 AM and 10 AM. A copulatory plug was sought immediately after mating, and the day that it was found was defined as day 0 of gestation.

Whole-embryo Culture
The methods that we used for removing and culturing embryos were originally established by New. At 7 AM on day 9 of gestation, rats were anesthetized with halothane, then killed by exsanguination. Uteri were excised and individual implantation sites were harvested into a sterile Petri dish containing Hank’s balanced salt solution. Egg cylinder were dissected from decidua under a dissecting microscope. The embryos were then divided into different stages of development according to a modified Theiler’s staging system, and only those at stage 10b (late primitive streak stage) were selected for culture. Reichert’s membrane was removed from the egg cylinder starting from the side opposite to the embryonic disc after the ectoplacental cone and roof of the ectoplacental cavity had been excised.

Embryos were randomly divided into groups as described below. Three to five embryos were placed in a single glass culture bottle (60 ml) that contained 1.5 ml per embryo of culture medium consisting of 50% pregnant rat serum, 50% male rat serum, and 20% Hank’s balanced salt solution; penicillin (100 U·ml⁻¹) and streptomycin (50 μg·ml⁻¹) were added to each bottle to prevent bacterial growth. The rat serum used was obtained from blood that had been centrifuged immediately after collection and heat-inactivated (56°C for 30 min). Bottles were flushed for 1 min with a gas mixture of either 5% O₂/5% CO₂/90% N₂, or 5% O₂/5% CO₂/75% N₂O/15% N₂, were capped with rubber stopcocks and rotated at 20 rpm in a 37–38°C incubator. After 24 h, all bottles were flushed with a gas mixture of 5% O₂/5% CO₂/90% N₂. Bottles were refilled with a gas mixture of 20% O₂/5% CO₂/75% N₂ at 3 PM on day 10, and with a gas mixture of 95% O₂/5% CO₂ at 6 AM on day 11. O₂, CO₂ and N₂O concentrations were monitored before and after each change of atmosphere by infrared gas spectrometry (Datex 254 airway monitor, Datex Medical Instrument, Tewksbury, MA), and recorded on a strip chart recorder.

At 10 AM on day 11 of gestation, culture was terminated and the crown-rump length and somite number of each embryo was determined. The size and shape of the head and body and the sitedness of the bulboventricular loop (heart), chorioallantoic placenta, and tail (lower part of the embryo) were recorded as in our previous study. Embryos were designated as having situs inversus when at least one of these three asymmetric body structures was opposite to normal.

Experiment 1
In the first experiment, we tested preventive effects of methionine, folic acid and prazosin (α₁-adrenergic antagonist) against N₂O-induced teratogenicity. Five groups of embryos were cultured as follows: (1) control (no treatment), (2) N₂O alone, (3) N₂O plus methionine (25 μg·ml⁻¹), (4) N₂O plus folic acid (5 μg·ml⁻¹), and (5) N₂O plus prazosin (10 μM). Concentrations of methionine and folic acid were chosen to be several-fold above normal serum levels for rats. The concentration of prazosin chosen was one that completely blocked phenylephrine (α₁-adrenergic agonist)-induced situs inversus in our previous studies. All chemicals were purchased from Sigma Chemical (St. Louis, MO).

Statistical analyses were performed as follows. Crown-rump length and number of somite pairs were compared among groups by one-way analysis of variance, and Fisher’s protected least significant difference test was used as an a posteriori test when differences were found with analysis of variance. Incidences of malformations and situs inversus were analyzed with a contingency table, and chi-squared analysis was used as an a posteriori test when there were differences. A posteriori tests was performed between each treatment group and both control and N₂O alone groups. A P value less than 0.05 was considered significant.

Experiment 2
Exposure to N₂O alone did not cause situs inversus in experiment 1. Based on the assumption that N₂O-induced α₁-adrenergic stimulation was not strong enough to cause situs inversus by itself, we decided to examine the effects of N₂O on the dose–response of phenylephrine-induced situs inversus. The incidences of situs inversus when embryos were exposed to different concentrations of phenylephrine were first determined. The study was then repeated with 0.5, 2.5, and 50 μM concentrations of phenylephrine in the presence of 75% N₂O. Based on the results, we selected 2.5 μM of phenylephrine to test whether prazosin blocks the additive effects of N₂O on the incidence of phenylephrine-induced situs inversus.

Statistical comparisons were performed among the incidences of situs inversus caused by phenylephrine...
alone, phenylephrine plus N₂O, and phenylephrine plus N₂O and prazosin with a contingency table. Chi-square analysis was used as an *a posteriori* test when there were differences. *P* value less than 0.05 was considered significant.

**Results**

*Experiment 1*

All 25 embryos in the control group developed normally in size and morphology (table 1). Treatment with N₂O alone resulted in an increased incidence of malformations (48.4%, *n* = 31), decreased crown-rump length and decreased number of somite pairs. Malformed embryos that were less affected had small heads but normal sized bodies, whereas embryos that were most affected had both malformed heads and bodies. In general, the abnormalities produced were similar to those that we reported in a previous study.*¹¹* Treatment with N₂O plus folinic acid or prazosin resulted in the same pattern of malformations (table 1 and fig. 1); that is, neither folinic acid nor prazosin conferred any protection. To the contrary, treatment with N₂O plus methionine resulted in almost no abnormalities (table 1 and fig. 1); that is, methionine almost completely protected the embryos from the effects of N₂O.

*Experiment 2*

Treatment with phenylephrine resulted in a dose-dependent increase in situs inversus that was unaccompanied by other malformations (table 2 and fig. 2). N₂O increased the incidence of situs inversus when co-administered with 0.5, 2.5 and 50 μM phenylephrine (table 2 and fig. 2). The increase for the 2.5 μM phenylephrine concentration was from 15.4% to 60.0% and was statistically significant (*P* < 0.05). Addition of 10 μM of prazosin to the N₂O/2.5 μM phenylephrine combination significantly (*P* < 0.05) decreased the incidence of situs inversus to 20.0% (*n* = 25).

**Discussion**

Currently, most investigators assume that N₂O-induced teratogenicity is due solely to N₂O's ability to

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**Table 1. Outcome for Embryos in Experiment 1**

<table>
<thead>
<tr>
<th></th>
<th>Embryos Studied (n)</th>
<th>Malformed Embryos (n)</th>
<th>Embryos with Situs Inversus (n)</th>
<th>Crown-Rump Length (mm, mean ± SD)</th>
<th>Somites Number (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>3.1 ± 0.1</td>
<td>22.8 ± 1.0</td>
</tr>
<tr>
<td>N₂O alone</td>
<td>31</td>
<td>15 (48.4)*</td>
<td>1 (4.0)</td>
<td>2.8 ± 0.2*</td>
<td>20.6 ± 1.5*</td>
</tr>
<tr>
<td>N₂O + methionine (25 μg/ml)</td>
<td>25</td>
<td>2 (8.0)†</td>
<td>1 (4.0)</td>
<td>3.1 ± 0.2†</td>
<td>22.9 ± 1.3†</td>
</tr>
<tr>
<td>N₂O + folinic acid (5 μg/ml)</td>
<td>25</td>
<td>13 (52.0)*</td>
<td>3 (12.0)</td>
<td>2.8 ± 0.2*</td>
<td>20.9 ± 1.3*</td>
</tr>
<tr>
<td>N₂O + prazosin (10 μM)</td>
<td>15</td>
<td>11 (73.3)*</td>
<td>0</td>
<td>2.5 ± 0.2*</td>
<td>13.7 ± 1.0*</td>
</tr>
</tbody>
</table>

*Values in parentheses are percentages.*

N₂O = nitrous oxide.

N₂O: 75% for the first 24 h of culture.

* *P* < 0.05 versus control.

† *P* < 0.05 versus N₂O alone.

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Table 2. Outcome for Embryos in Experiment 2

<table>
<thead>
<tr>
<th>Phenylephrine (μM)</th>
<th>Embryos Studied (n)</th>
<th>Malformed Embryos (n)</th>
<th>Embryos with Situs Inversus (n)</th>
<th>Crown–Rump Length (mm, mean ± SD)</th>
<th>Somites Number (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>25</td>
<td>0</td>
<td>1 (4.0)</td>
<td>3.0 ± 0.2</td>
<td>22.9 ± 0.9</td>
</tr>
<tr>
<td>2.5</td>
<td>26</td>
<td>0</td>
<td>4 (15.4)</td>
<td>3.1 ± 0.1</td>
<td>23.1 ± 0.9</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>0</td>
<td>9 (37.5)</td>
<td>3.2 ± 0.1</td>
<td>23.2 ± 0.9</td>
</tr>
<tr>
<td>50</td>
<td>26</td>
<td>0</td>
<td>14 (53.8)</td>
<td>3.1 ± 0.2</td>
<td>23.2 ± 0.9</td>
</tr>
<tr>
<td>N₂O + phenylephrine (μM)</td>
<td>25</td>
<td>20 (80.0)</td>
<td>4 (16.0)</td>
<td>2.6 ± 0.2</td>
<td>20.1 ± 1.4</td>
</tr>
<tr>
<td>0.5</td>
<td>24</td>
<td>18 (75.0)</td>
<td>15 (60.0)*</td>
<td>2.7 ± 0.2</td>
<td>20.9 ± 1.1</td>
</tr>
<tr>
<td>2.5</td>
<td>35</td>
<td>28 (60.0)</td>
<td>22 (62.9)</td>
<td>2.7 ± 0.2</td>
<td>21.2 ± 1.2</td>
</tr>
<tr>
<td>N₂O + prazosin (10 μM) + phenylephrine (μM)</td>
<td>25</td>
<td>16 (64.0)</td>
<td>5 (20.0)†</td>
<td>2.7 ± 0.2</td>
<td>21.2 ± 0.9</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
N₂O = nitrous oxide.
N₂O: 75% for the first 24 h of culture.
* P < 0.05 versus 2.5 μM phenylephrine.
† P < 0.05 versus N₂O + 2.5 μM phenylephrine.

Phenylephrine (PH) oxidize vitamin B₁₂ which cannot then function as a coenzyme for methionine synthase. This enzyme catalyzes the transmethylation from methyltetrahydrofolate and homocysteine to produce tetrahydrofolate and methionine (fig. 3). The expected result of its inhibition is decreased tetrahydrofolate which leads to decreased thymidylate (thymidine monophosphate) and hence impaired DNA synthesis, and decreased methionine which leads to impaired methylation reactions. Significant inactivation of methionine synthase by N₂O occurs rapidly in animals and humans, and is known to cause a pernicious anemia-like syndrome consisting of subacute combined degeneration of the spinal cord, megablastic anemia and pancytopenia in humans. Because the hematologic changes in humans are prevented by folic acid (5-formyl tetrahydrofo-

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Fig. 2. Dose–response for phenylephrine (PH)-induced situs inversus, and the effects of N₂O alone and N₂O plus prazosin (PR). *Value significantly higher than control: P < 0.05. #Value significantly less than N₂O alone: P < 0.05.

Fig. 3. Pathway of inhibition of methionine synthase by N₂O and its potential metabolic consequences. SAM = S-adenosylmethionine; THF = tetrahydrofolate.
late) administered with N₂O, presumably because DNA synthesis is restored to normal (fig. 3). Impairment of DNA synthesis has been proposed to account for N₂O-induced teratogenicity. Such thinking has even led to the recommendation that folic acid be administered to all pregnant women having an operation during which N₂O is likely to be administered.‡

Some support for the idea that decreased DNA synthesis is the most important factor in N₂O-induced teratogenicity came from a study in which folic acid was said to prevent such an effect in rats. However, after carefully reanalyzing their data, we concluded that preventive effects of folic acid were limited to partial protection of minor skeletal abnormalities. This conclusion has been confirmed in a similar in vivo study performed in our laboratory and in the current in vitro study. It is also consistent with our previous findings that maximum reduction of methionine synthase activity and decrease in DNA synthesis occur at concentrations of N₂O that are well below those that produce teratogenicity. It is not clear at this time why decreased tetrahydrofolate and DNA synthesis play almost no role in N₂O-induced teratogenicity. Certainly, N₂O’s effect on DNA synthesis is only partial. Furthermore, it is likely that the salvage pathway for thymidylate, which is known to exist in many mammalian tissues, becomes more active in the embryo to compensate for the decreased de novo synthesis of thymidylate.

Until now, the role of methionine deficiency on N₂O-induced teratogenicity has been neglected. Our results suggest for the first time that it plays the major role in N₂O-induced teratogenicity. Other investigators have reported that embryos grown in methionine deficient culture medium do not develop normally, although the abnormalities produced were not exactly the same as those seen in the current study. Their and our results are not surprising because methionine, via its activated form S-adenosylmethionine, is the principal substrate for methylation in many biochemical reactions. Nevertheless, whether impaired methylation or some other mechanism is involved in N₂O-induced teratogenicity remains to be determined.

During the past few years we have become increasingly interested in whether some aspects of N₂O’s reproductive toxicity could relate to its sympathomimetic actions. Such actions of N₂O on embryos have not been investigated, but it is well known that N₂O causes central and peripheral sympathetic stimulation in both mature animals and humans, and that these lead to marked physiologic changes, especially of the cardiovascular system. The precise cellular mechanisms by which N₂O produces these sympathetic effects are unknown although their elucidation would be helpful in the clinical management of anesthetized patients. It is known, however, that N₂O administration increases urinary catecholamine levels, plasma norepinephrine concentrations, and peripheral vascular resistance in humans. N₂O also has been shown to increase sympathetic effenter nerve activity to vessels of skeletal muscle. Using the whole-embryo culture system, we have recently demonstrated that stimulation of α₁ but not of α₂ or β adrenergic stimulation caused situs inversus without causing other abnormalities. In the current study, situs inversus did not occur with N₂O unlike in our previous studies in which rats were obtained from different sources. Nevertheless, we have demonstrated that N₂O has an additive effect on phenylephrine-induced situs inversus which is blocked by prazosin. Results from the current and previous studies suggest that N₂O stimulates α₁-adrenergic receptors in the embryo, but that the effects are weak and will only result in situs inversus in susceptible animals.

In summary, using a rat whole-embryo culture system, we have demonstrated that supplemental methionine, but not folic acid, almost completely prevents N₂O-induced teratogenicity. Our results suggest for the first time that decreased methionine rather than tetrahydrofolate plays the major role in N₂O-induced teratogenicity other than situs inversus. We also have demonstrated that N₂O stimulates α₁-adrenergic receptors in the embryo and may cause situs inversus in susceptible animals. Clearly, N₂O-induced teratogenicity is multifactorial. The detailed molecular mechanisms involved in the production of reproductive toxicity by lack of methionine and of situs inversus by α₁-adrenergic stimulation remain to be determined.

References


MECHANISMS OF N₂O-INDUCED TERATOGENICITY


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