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

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

Background: **Nitrous oxide (N_2O)–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 N_2O.**

Methods: **A rat whole-embryo culture system was used to determine whether N_2O-induced teratogenicity can be prevented with supplemental methionine or folinic acid and whether N_2O-induced situs inversus is mediated by α_1-adrenergic stimulation. Embryos were explanted on day 9 of gestation, and those at stage 10b (late primitive streak stage) were cultured with or without N_2O and the various chemicals, methionine (25 $\mu g \cdot ml^{-1}$), folinic acid (5 $\mu g \cdot ml^{-1}$), phenylephrine (range 0.5–50 μM) and prazosin (10 μM). Embryos in the N_2O 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 N_2O alone resulted in increased incidences of malformations and growth retardation. Methionine, but not folinic acid or prazosin, almost completely prevented N_2O-induced malformations and growth retardation. N_2O 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 N_2O-induced teratogenicity in rats. They also indicate that N_2O stimulates the α_1-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.)**

* Research Associate, Stanford University School of Medicine and Palo Alto Veterans Affairs Medical Center.

† Professor of Anesthesia, Stanford University School of Medicine; Chief of Anesthesiology Service, Palo Alto Veterans Affairs Medical Center.

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Address reprint requests to Dr. Fujinaga: 3801 Miranda Avenue, 112A, Palo Alto, California 94304.

IN the late 1960s, Fink and Shepard[1,2] published their seminal work on the teratogenicity of nitrous oxide (N_2O) 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 N_2O 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 N_2O.[4,5] The protective effects of methionine supplementation has not been investigated. More recently, the sympathomimetic effects of N_2O have also received attention as a possible cause of some malformations, especially situs inversus which is known to be caused by α_1-adrenergic agonists.[6–8]

We have established a rat whole-embryo culture model to assist in examining the mechanisms of N_2O-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 N_2O 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 N_2O-induced teratogenicity, and to determine whether N_2O-induced situs inversus is mediated by α_1-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.[20] 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.[21] 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,[22,23] 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.[24]

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 30% pregnant rat serum, 50% male rat serum, and 20% Hank's balanced salt solution; penicillin (100 $U \cdot ml^{-1}$) and streptomycin (50 $\mu g \cdot ml^{-1}$) 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% O_2/5% CO_2/90% N_2, or 5% O_2/5% CO_2/75% N_2O/15% N_2, 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% O_2/5% CO_2/90% N_2. Bottles were reflushed with a gas mixture of 20% O_2/5% CO_2/75% N_2 at 3 PM on day 10, and with a gas mixture of 95% O_2/5% CO_2 at 6 AM on day 11. O_2, CO_2 and N_2O 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 sidedness of the bulboventricular loop (heart), chorioallantoic placenta, and tail (lower part of the embryo) were recorded as in our previous study.[11] 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, folinic acid and prazosin (α_1-adrenergic antagonist) against N_2O-induced teratogenicity. Five groups of embryos were cultured as follows: (1) control (no treatment), (2) N_2O alone, (3) N_2O plus methionine (25 $\mu g \cdot ml^{-1}$), (4) N_2O plus folinic acid (5 $\mu g \cdot ml^{-1}$), and (5) N_2O plus prazosin (10 μM). Concentrations of methionine[25,26] and folinic acid[27] were chosen to be several-fold above normal serum levels for rats. The concentration of prazosin chosen was one that completely blocked phenylephrine (α_1-adrenergic agonist)-induced situs inversus in our previous studies.[6,8] 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 N_2O alone groups. A P value less than 0.05 was considered significant.

Experiment 2

Exposure to N_2O alone did not cause situs inversus in experiment 1. Based on the assumption that N_2O-induced α_1-adrenergic stimulation was not strong enough to cause situs inversus by itself, we decided to examine the effects of N_2O 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% N_2O. Based on the results, we selected 2.5 μM of phenylephrine to test whether prazosin blocks the additive effects of N_2O on the incidence of phenylephrine-induced situs inversus.

Statistical comparisons were performed among the incidences of situs inversus caused by phenylephrine

alone, phenylephrine plus N_2O, and phenylephrine plus N_2O 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 N_2O 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 N_2O 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 N_2O plus methionine resulted in almost no abnormalities (table 1 and fig. 1); that is, methionine almost completely protected the embryos from the effects of N_2O.

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). N_2O increased the incidence of situs inversus when coadministered 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 N_2O/2.5 μM phenylephrine combination significantly ($P < 0.05$) decreased the incidence of situs inversus to 20.0% (n = 25).

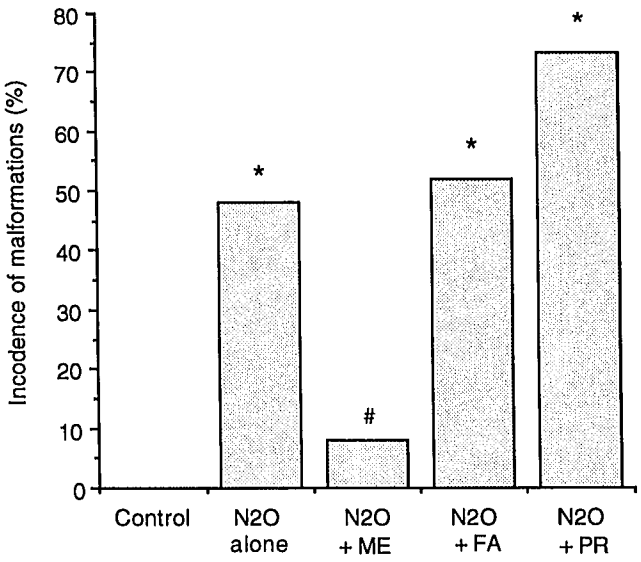

Fig. 1. Incidence of malformations induced by N_2O alone and N_2O plus methionine (ME), folinic acid (FA), or prazosin (PR). *Value significantly higher than control: $P < 0.05$. #Value significantly less than N_2O alone: $P < 0.05$.

Discussion

Currently, most investigators assume that N_2O-induced teratogenicity is due solely to N_2O's ability to

Table 1. Outcome for Embryos in Experiment 1

	Embryos Studied (n)	Malformed Embryos (n)	Embryos with Situs Inversus (n)	Crown–Rump Length (mm, mean ± SD)	Somites Number (mean ± SD)
Control	25	0	0	3.1 ± 0.1	22.8 ± 1.0
N_2O alone	31	15 (48.4)*	1 (4.0)	2.8 ± 0.2*	20.6 ± 1.5*
N_2O + methionine (25 μg/ml)	25	2 (8.0)†	1 (4.0)	3.1 ± 0.2†	22.9 ± 1.3†
N_2O + folinic acid (5 μg/ml)	25	13 (52.0)*	3 (12.0)	2.8 ± 0.2*	20.9 ± 1.3*
N_2O + prazosin (10 μM)	15	11 (73.3)*	0	2.5 ± 0.2*	19.7 ± 1.0*

Values in parentheses are percentages.

N_2O = nitrous oxide.

N_2O: 75% for the first 24 h of culture.

* $P < 0.05$ *versus* control.

† $P < 0.05$ *versus* N_2O alone.

Table 2. Outcome for Embryos in Experiment 2

	Embryos Studied (n)	Malformed Embryos (n)	Embryos with Situs Inversus (n)	Crown–Rump Length (mm, mean ± SD)	Somites Number (mean ± SD)
Phenylephrine (μM)					
0.5	25	0	1 (4.0)	3.0 ± 0.2	22.9 ± 0.9
2.5	26	0	4 (15.4)	3.1 ± 0.1	23.1 ± 0.9
5	24	0	9 (37.5)	3.2 ± 0.1	23.2 ± 0.9
50	26	0	14 (53.8)	3.1 ± 0.2	23.2 ± 0.9
N_2O + phenylephrine (μM)					
0.5	25	20 (80.0)	4 (16.0)	2.6 ± 0.2	20.1 ± 1.4
2.5	24	18 (75.0)	15 (60.0)*	2.7 ± 0.2	20.9 ± 1.1
50	35	28 (80.0)	22 (62.9)	2.7 ± 0.2	21.2 ± 1.2
N_2O + prazosin (10 μM) + phenylephrine (μM)					
2.5	25	16 (64.0)	5 (20.0)†	2.7 ± 0.2	21.2 ± 0.9

Values in parentheses are percentages.

N_2O = nitrous oxide.

N_2O: 75% for the first 24 h of culture.

* $P < 0.05$ *versus* 2.5 μM phenylephrine.

† $P < 0.05$ *versus* N_2O + 2.5 μM phenylephrine.

oxidize vitamin B_{12} which cannot then function as a coenzyme for methionine synthase.[3] 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 N_2O occurs rapidly in animals[28,29] and humans,[30] and is known to cause a pernicious anemia-like syndrome consisting of subacute combined degeneration of the spinal cord,[31] megaloblastic anemia and pancytopenia[32] in humans. Because the hematologic changes in humans are prevented by folinic acid (5-formyl tetrahydrofo-

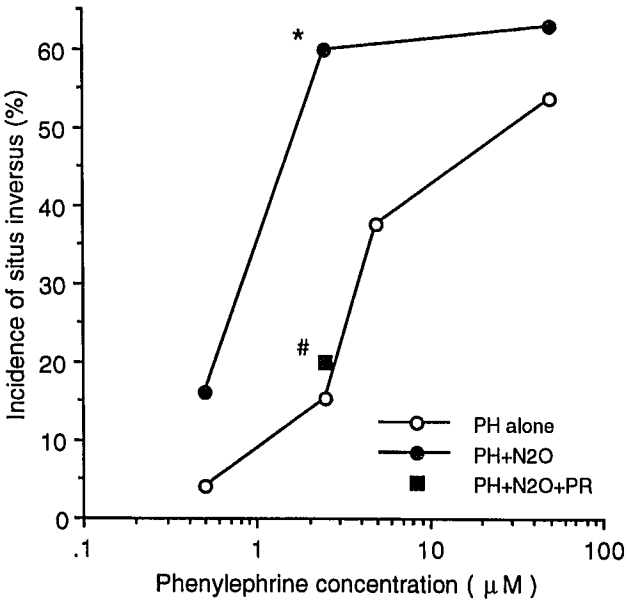

Fig. 2. Dose–response for phenylephrine (PH)-induced situs inversus, and the effects of N_2O alone and N_2O plus prazosin (PR). *Value significantly higher than control: $P < 0.05$. #Value significantly less than N_2O alone: $P < 0.05$.

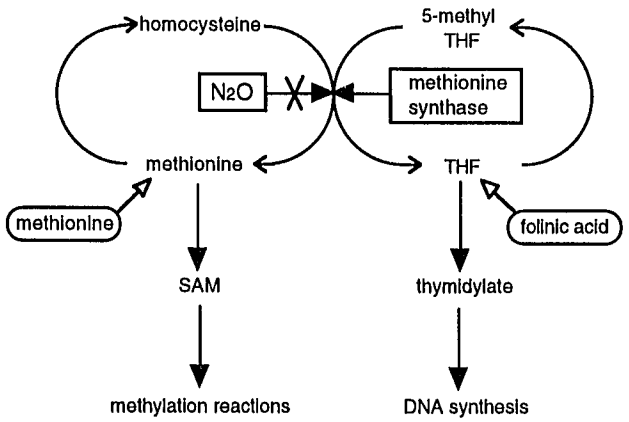

Fig. 3. Pathway of inhibition of methionine synthase by N_2O and its potential metabolic consequences. SAM = S-adenosylmethionine; THF = tetrahydrofolate.

late) administered with N_2O, presumably because DNA synthesis is restored to normal (fig. 3),[33] impairment of DNA synthesis has been proposed to account also for N_2O-induced teratogenicity.[34] Such thinking has even led to the recommendation that folinic acid be administered to all pregnant women having an operation during which N_2O is likely to be administered.‡

Some support for the idea that decreased DNA synthesis is the most important factor in N_2O-induced teratogenicity came from a study in which folinic acid was said to prevent such an effect in rats.[4] However, after carefully reanalyzing their data, we concluded that preventive effects of folinic 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[5] 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 N_2O that are well below those that produce teratogenicity.[35,36] It is not clear at this time why decreased tetrahydrofolate and DNA synthesis play almost no role in N_2O-induced teratogenicity. Certainly, N_2O's effect on DNA synthesis is only partial.[35,36] 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 N_2O-induced teratogenicity has been neglected. Our results suggest for the first time that it plays the major role in N_2O-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.[25,26] 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 N_2O-induced teratogenicity remains to be determined.

During the past few years we have become increasingly interested in whether some aspects of N_2O's reproductive toxicity could relate to its sympathomimetic actions. Such actions of N_2O on embryos have not been investigated, but it is well known that N_2O 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.[37,38] The precise cellular mechanisms by which N_2O produces these sympathetic effects are unknown although their elucidation would be helpful in the clinical management of anesthetized patients. It is known, however, that N_2O administration increases urinary catecholamine levels, plasma norepinephrine concentrations, and peripheral vascular resistance in humans.[39] N_2O also has been shown to increase sympathetic efferent nerve activity to vessels of skeletal muscle.[40,41] Using the whole-embryo culture system, we have recently demonstrated that stimulation of α_1 but not of α_2 or β adrenergic stimulation caused situs inversus without causing other abnormalities.[6–8] In the current study, situs inversus did not occur with N_2O unlike in our previous studies in which rats were obtained from different sources.[10,11] Nevertheless, we have demonstrated that N_2O has an additive effect on phenylephrine-induced situs inversus which is blocked by prazosin. Results from the current and previous studies suggest that N_2O stimulates α_1-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 folinic acid, almost completely prevents N_2O-induced teratogenicity. Our results suggest for the first time that decreased methionine rather than tetrahydrofolate plays the major role in N_2O-induced teratogenicity other than situs inversus. We also have demonstrated that N_2O stimulates α_1-adrenergic receptors in the embryo and may cause situs inversus in susceptible animals. Clearly, N_2O-induced teratogenicity is multifactorial. The detailed molecular mechanisms involved in the production of reproductive toxicity by lack of methionine and of situs inversus by α_1-adrenergic stimulation remain to be determined.

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