Anesthesiology
69:401–404, 1988

Rat Whole Embryo Culture: An In Vitro *Model for Testing Nitrous Oxide Teratogenicity*

Masahiko Fujinaga, M.D., Richard I. Mazze, M.D.,† Jeffrey M. Baden, M.D.,‡ Alan G. Fantel, Ph.D.,§ Thomas H. Shepard, M.D.¶*

The teratogenic effects of nitrous oxide (N_2O) on postimplantation rat embryos were studied using a whole embryo culture system to separate the direct effects of N_2O from those that are maternally mediated. A total of 100, 10-day-old rat embryos were cultured in either a control atmosphere (75% N_2, 20% O_2, and 5% CO_2), or a N_2O atmosphere (75% N_2O, 20% O_2, and 5% CO_2). After 22 h of culture embryos were examined microscopically, and protein and DNA contents were determined. DNA content was significantly lower in the embryos exposed to N_2O compared with the controls. Additionally, three malformed embryos and four embryos with left-sided tails were observed in the N_2O group, whereas no abnormalities were observed in the control group. There were no differences in crown-rump length, somite numbers, limb bud index, and protein content between the two groups of embryos. The positive findings in this study indicate that whole embryo culture is useful for studying the mechanisms of N_2O teratogenicity. (Key words: Anesthetics: nitrous oxide. Culture: whole embryo culture. Pregnancy: toxicity.)

IN 1967 Fink *et al.* showed that N_2O was teratogenic in Sprague-Dawley rats.[1] Since then the *in vivo* model of N_2O teratogenicity in rats has become well established; continuous exposure to at least 50% N_2O for 24 hours during organogenesis results in increased incidences of fetal wastage, major visceral anomalies, *e.g.*, right sided aortic arch, and major and minor skeletal malformations.[2–6] However, *in vivo* studies have several drawbacks, *e.g.*, they are expensive and time-consuming, and they cannot be used to separate direct teratogenic and biochemical effects from those that are maternally mediated. *In vitro* whole embryo culture overcomes these limitations, but it is not known whether N_2O exposure would produce abnormalities in such a system. We performed the present study to determine if *in vitro* whole embryo culture offered such a possibility.

Methods

Twelve timed-pregnant Sprague-Dawley rats were obtained from the breeder (Tyler Laboratories, Bellevue, WA) between day 4 and day 6 of pregnancy. (Day 0 of pregnancy was defined as the day when a copulatory plug was observed in the vagina.) They had *ad libitum* food (Purina Lab Chow) and water and were provided artificial lighting on a 14-h light/10-h dark cycle; room temperature was kept constant at 21° C. The explanation of embryos and the culture procedures we used were originally described by New[7] and subsequently modified by Mirkes *et al.*[8] Briefly, on the morning of day 10 of pregnancy, rats were anesthetized with diethyl ether, laparotomy was performed, and the uterus was excised and placed in a sterile petri dish containing Hank's balanced salt solution (HBSS). The individual egg cylinders were then removed from the uterus, and maternal decidua and Reichert's membrane were dissected away from the embryo using a dissecting microscope (fig. 1). Twenty to 30 viable embryos of approximately 10 somites were obtained in this fashion and were randomly divided into control and treatment groups. In addition, blood was collected from the abdominal aorta, centrifuged at 5° C for 20 min, and the serum decanted and then frozen at −20° C for later usage as culture medium for the embryos. Residual diethyl ether in the serum was eliminated by heating serum to 56° C for 20 min.

Groups of 10–15 embryos were cultured in a single glass bottle (approximate volume, 125 ml) that contained 7 ml each of heat-inactivated (56° C for 20 min) human and rat serum. Penicillin (100 $U \cdot ml^{-1}$) and streptomycin (50 $\mu g \cdot ml^{-1}$) were added to each bottle to prevent bacterial growth.[8] After the embryos were placed in the appropriate culture bottles, the bottles

* Research Fellow in Anesthesia, Stanford University School of Medicine and Palo Alto VA Medical Center; Research Associate in Pediatrics, Central Laboratory for Human Embryology.

† Professor of Anesthesia and Associate Chairman, Stanford University School of Medicine; Staff Anesthesiologist, Palo Alto VA Medical Center.

‡ Associate Professor of Anesthesia, Stanford University School of Medicine; Chief of Anesthesiology Service, Palo Alto VA Medical Center.

§ Associate Professor of Pediatrics, Central Laboratory for Human Embryology.

¶ Professor of Pediatrics and Chief of Central Laboratory for Human Embryology.

Received from the Department of Anesthesia, Stanford University School of Medicine, Stanford, California; Anesthesiology Service, Palo Alto VA Medical Center, Palo Alto, California; and the Central Laboratory for Human Embryology, Department of Pediatrics, University of Washington, Seattle, Washington. Accepted for publication April 12, 1988. Supported by the Veterans Administration, the Anesthesia/Pharmacology Research Foundation, and NIH Grant HD12717.

Address reprints requests to Dr. Mazze: Department of Anesthesia, Stanford University School of Medicine, Stanford, California 94305.

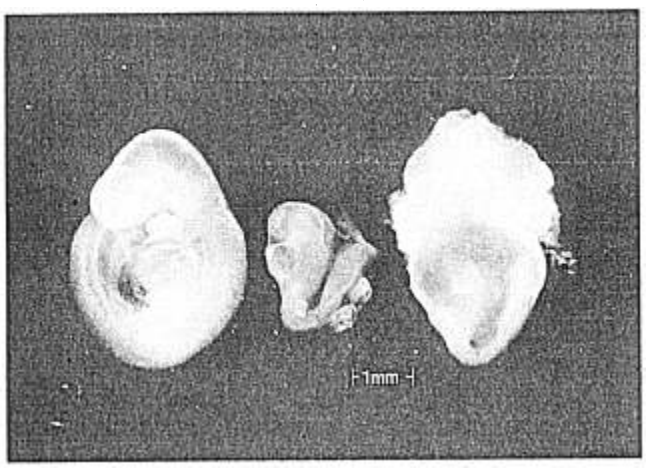

FIG. 1. *Right.* Day 10 embryo before culture is shown surrounded by the yolk sac with the placental cone at the top. The embryo faces to the left and the tail is dorsally flexed. *Center.* Day 10 embryo facing left with yolk sac removed. The bulge below the head is the primitive heart. Embryo at this stage has approximately 10 somites. *Left.* Day 11 embryo after culture with yolk sac and amnion removed showing the usual right-sided position of the ventrally flexed tail.

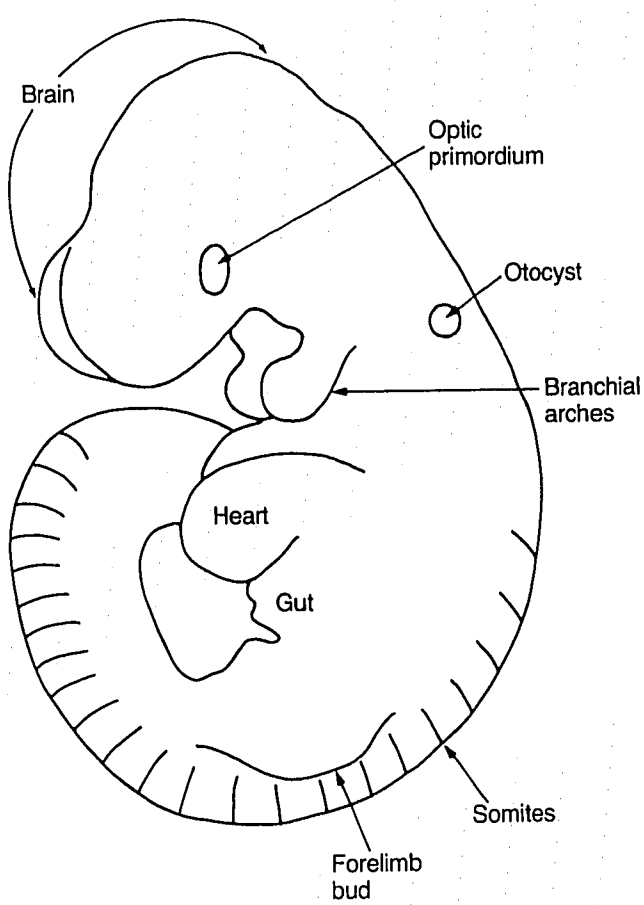

FIG. 2. Sketch of day 11 embryo after culture.

TABLE 1. Morphologic and Biochemical Data (mean ± SD) after Embryo Culture (day 11)

	Control	N_2O
No. of embryos studied	46	54
Crown-rump length (mm)	3.7 ± 0.3	3.5 ± 0.3
No. of somites	25.0 ± 1.2	24.3 ± 1.9
Limb bud index	21.8 ± 0.4	21.7 ± 0.7
DNA content ($\mu g \cdot embryo^{-1}$)	55.0 ± 10.7	43.7 ± 11.1*
Protein content ($\mu g \cdot embryo^{-1}$)	439 ± 105	399 ± 137

* $P < 0.05$.

were flushed with a gas mixture, which consisted of either 75% N_2, 20% O_2, and 5% CO_2 (control group) or 75% N_2O, 20% O_2, and 5% CO_2 (N_2O group), for 20 min at 37° C. Culture bottles were then tightly capped, sealed with parafilm, and placed in a 37° C incubator to rotate at 20–40 rpm.

After an average of 22 h of incubation the embryos were removed from the culture bottle and placed in a petri dish containing HBSS. Using a dissecting microscope, the number of somites, crown-rump length, limb bud index,[9] and presence of malformations were immediately determined for each embryo without knowledge of treatment groups. (Embryonic parts are shown in fig. 2.) Approximately 25 embryos could be examined in a 2-h period. Thus, to maintain a relatively uniform incubation period the experiment was replicated four times. After the embryos were examined, they were stored individually in 1 ml of 0.1 M sodium phosphate buffer (*p*H 7.4), and frozen at −20° C for subsequent analyses of protein and DNA content. Protein was assayed according to the methods of Bradford[10] using bovine gamma globulin as a standard. DNA was assayed according to the method of Labarca and Paigen[11] using calf thymus DNA as a standard. Statistical analyses were performed by Student's *t* test and Fisher's exact test, as appropriate; $P < 0.05$ was considered significant.

Results

A total of 100 embryos were cultured in the four replicate experiments; data from groups treated similarly were pooled. There were no differences between the control and N_2O-treated embryos in crown-rump length, number of somites, and limb bud index (table 1). The mean DNA content was 20.5% lower ($P < 0.001$) in the N_2O-treated embryos (43.7 ± 11.1 $\mu g \cdot embryo^{-1}$) than in the controls (55.0 ± 10.7 $\mu g \cdot embryo^{-1}$). The mean protein content was 9.3%

lower in embryos exposed to N_2O (399 ± 137 $\mu g \cdot embryo^{-1}$) than in the controls (439 ± 105 $\mu g \cdot embryo^{-1}$), but this difference was not statistically significant ($P = 0.10$). Seven embryos among the 54 exposed to N_2O were abnormal compared with none in the control group ($P = 0.02$ by Fisher's exact test). Of these, three had abnormally kinked tails and low mean DNA (26.0 $\mu g \cdot embryo^{-1}$) and protein (211 $\mu g \cdot embryo^{-1}$) contents; one of the three also had a severe head malformation (DNA content, 23 μg; protein content, 172 μg). The other four abnormal embryos exposed to N_2O (mean DNA content, 44.3 μg; mean protein content, 443 μg) had left-sided tails, as opposed to the usual right-sided position of the tail on day 11.

Discussion

It has been proposed that N_2O is teratogenic because it inhibits methionine synthase activity in the developing embryos.[12,13] This leads to interference with DNA production and, thus, to abnormal development. This theory is based on the original work of Banks *et al.*,** who demonstrated that N_2O oxidized the cobalt in vitamin B_{12} from the active cob(I)alamin form to the inactive cob(II)alamin form. Recently, we have shown that 1/4 MAC isoflurane or halothane administered with N_2O prevented teratogenic effects in rats but did not prevent inhibition of methionine synthase activity.[6,14] However, treatment with folinic acid, which should reverse the effects of N_2O administration on DNA production, did not prevent N_2O teratogenicity.[14] Our results suggest that inhibition of methionine synthase activity does not entirely explain N_2O teratogenicity and that other mechanisms must also be operative.

To further probe mechanisms of N_2O teratogenicity, we sought a testing system that would eliminate maternal factors and would examine only the direct effects of N_2O on the developing embryo. *In vitro* whole embryo culture would be useful for this purpose providing biochemical and morphologic differences could be demonstrated between embryos exposed to a control atmosphere and those exposed to N_2O. This proved to be the case. Our findings of morphologic abnormalities and of reduced DNA content indicate that at least part of the teratogenic effect of N_2O is independent of its maternal physiologic and biochemical actions. They do not establish, however, that these direct effects are due to inhibition of methionine synthase or decreased DNA synthesis.

For the current study one problem that must be avoided is the use of a culture medium containing excess of methionine, s-adenosylmethionine, and folates, which may mask the effects of N_2O. For that reason we have used only serum in our culture experiments that would not contain an excess of these substances. Indeed, the folates in serum are almost entirely in the form of methyltetrahydrofolate, which cannot reverse the decreased synthesis of DNA produced by N_2O-induced inhibition of methionine synthase.

In vitro whole embryo culture has been used in other laboratories to elucidate mechanisms of normal and abnormal development.[15,16] Studies are usually performed with rat embryos, which can be grown in culture during all of the major events of organogenesis with the possible exception of palatal fusion, which occurs on day 16. It is common to explant embryos at about the 10 somite stage (day 10) and culture them for 1–2 days. Early on day 10 the heartbeat is established and later on day 10 the neural tube begins to close. Successful embryo culture generally is not possible after day 12 when *in vivo* the chorioallantoic placenta assumes the prominent nutritive role. It also is possible to study the development of biochemical process in the *in vitro* explanted developing embryos. For example, glucose utilization and lactate production have been examined extensively. It is known that the day 10 embryos are dependent on the Embden-Meyerhoff glycolytic pathway for their energy, whereas on days 11–12, the Krebs cycle and oxidative phosphorylation predominate.[17]

In summary, we have shown that N_2O produces direct biochemical and morphologic defects in developing embryos. The whole embryo culture system that we used should be of value for studying mechanisms of N_2O teratogenicity.

References

1. Fink BR, Shepard TH, Blandau RJ: Teratogenic activity of nitrous oxide. Nature 214:146–148, 1967
2. Shepard TH, Fink BR: Teratogenic activity of nitrous oxide in rats, Toxicity of Anesthetics. Edited by Fink BR. Baltimore, Williams & Wilkins, 1968, pp 308–323
3. Lane GA, Nahrwold ML, Tait AR, Taylor-Busch M, Cohen PJ, Beaudoin AR: Nitrous oxide is fetotoxic, xenon is not. Science 210:899–901, 1980
4. Mazze RI, Wilson AI, Rice SA, Baden JM: Reproduction and fetal development in rats exposed to nitrous oxide. Teratology 30:259–265, 1984
5. Mazze RI, Fujinaga M, Baden JM: Reproductive and teratogenic effects of nitrous oxide, fentanyl and their combination in Sprague-Dawley rats. Br J Anaesth 59:1291–1297, 1987

** Banks RGS, Henderson RJ, Pratt JM: Reactions of gases in solution. Part III. Some reactions of nitrous oxide with transition-metal complexes. J Chem Soc A2886, 1968.

6. Fujinaga M, Baden JM, Yhap EO, Mazze RI: Reproductive and teratogenic effects of nitrous oxide, isoflurane and their combination in Sprague-Dawley rats. ANESTHESIOLOGY 67:960–964, 1987
7. New DAT: Whole embryo culture and the study of mammalian embryos during organogenesis. Biol Rev 53:81–122, 1978
8. Mirkes PE, Greenway JC, Rogers JM, Brundrett RB: Role of acrolein in cyclophosphamide teratogenicity in rat embryo in vitro. Toxicol Appl Pharmacol 72:281–291, 1984
9. Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. J Morphol 88:49–92, 1951
10. Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254, 1976
11. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102:344–352, 1980
12. Nunn JF, Chanarin I: Nitrous oxide inactivates methionine synthase, Nitrous Oxide/N_2O. Edited by Eger EI. New York, Elsevier, 1985, pp 211–233
13. Nunn JF: Clinical aspects of the interaction between nitrous oxide and vitamin B_{12}. Br J Anaesth 59:3–13, 1987
14. Mazze RI, Fujinaga M, Baden JM: Halothane prevents nitrous oxide teratogenicity in Sprague-Dawley rats; Folinic acid does not. Teratology, in press
15. Flynn TJ: Teratological research using in vitro systems. I. Mammalian whole embryo culture. Environ Health Perspect 72:203–210, 1987
16. Shepard TH, Fantel AG, Mirkes PE: Somite-stage mammalian embryo culture: Use in study of normal physiology and mechanisms of teratogenesis, Banbury Report 26: Developmental Toxicology: Mechanisms and Risk. New York, Cold Spring Harbor Laboratory, 1987, pp 29–44
17. Shepard TH, Tanimura T, Robkin M: Energy metabolism in early mammalian embryos. Dev Biol 4(Suppl):42–58, 1970