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## Heat- and Alcohol-Induced Neural Tube Defects: Interactions with Folate in a Golden Hamster Model

JOHN M. GRAHAM, JR. AND VERGIL H. FERM

*Dysmorphology Unit, Department of Maternal and Child Health [J.M.G.], and Department of Anatomy, Dartmouth Medical School [V.H.F.], Hanover, New Hampshire 03756*

**ABSTRACT.** Maternal hyperthermia or ethanol each can induce fetal neural tube defects (NTD) following exposures on the 8th day of gestation in golden hamsters. To explore the relationship between NTD and varying doses of either heat or ethanol, timed pregnant golden hamsters were exposed to various doses of either 25% ethanol, or heat in an incubator at 39.5° C on the morning of the 8th day of gestation. Two doses of 0.015 ml/g body weight of 25% ethanol 4 h apart resulted in a 44% incidence of NTD when fetuses were examined on day 13. Single doses of 25% ethanol (either 0.015 or 0.0075 ml/g) resulted in very low incidences of NTD that were not significantly different from zero. A 50-min exposure to heat resulted in a 35% incidence of NTD. A shorter exposure (44 min) resulted in a 23% incidence, and a longer exposure (56 min) resulted in a 68% incidence of NTD. A 0.0075 ml/g dose of 25% ethanol, followed by these same durations of heat, resulted in incidences of NTD that were not significantly different from heat alone. In order to determine what effect folate supplementation might have on ethanol- or heat-induced

NTD, osmotic pumps filled with either folate or saline were placed subcutaneously in pregnant hamsters on the 6th day of gestation. Animals were then exposed to ethanol or heat on the morning of day 8. No significant protection from NTD was evident among fetuses from mothers supplemented with folate despite significant elevations in their red cell folate levels on day 8 of gestation. (*Pediatr Res* 19: 247-251, 1985)

### Abbreviation

NTD, neural tube defect(s)

Experimental results from several animal species suggest that hyperthermia during the time of neural tube closure may lead to NTD (7, 8, 15, 26). Furthermore, epidemiologic studies suggest the human embryo is not immune to such effects (2, 10, 14, 21). Such studies suggest that as many as 10% of live births and 14% of human abortuses with NTD may have been exposed to hyperthermia during early gestation (25). Other studies have documented frequent occurrences of anencephaly following epidemics of febrile illnesses such as influenza (5, 6, 13). Similarly, experimental studies suggest that exposure to high doses of

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Address for Reprints John M. Graham, Jr., M.D., Sc.D., Dysmorphology Unit, Department of Maternal and Child Health, Dartmouth Medical School, Hanover, NH 03756.

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ethanol during the time of neural tube closure results in a significantly increased incidence of NTD (3, 16, 22, 31, 33). A number of anecdotal human case reports also suggest that NTD are among the central nervous system malformations that have been observed in the wake of heavy drinking during early gestation (4, 11, 12, 20). Other workers have suggested that perinatal vitamin supplementation, particularly with folate, might be effective in preventing NTD recurrences (18, 28, 30).

We have shown that NTD are readily induced by heat in the hamster model system (15). In addition, in the present study we show that ethanol is capable of inducing NTD in the hamster, confirming similar results in mice and chickens (3, 16, 22, 28, 31, 33). If folate supplementation affords protection from teratogen-induced NTD, then it should reduce the incidence of NTD induced by either heat or alcohol in such an experimental animal system. Herein we report the effect of folate supplementation prior to and during the stages of critical embryogenesis for neural tube closure in the golden hamster.

#### METHODS

In the golden hamster, neural tube closure occurs between 8.00 h on day 8 and 8.00 h on day 9, counting the first postconceptional day as day 1 (9). Timed pregnant golden hamsters were exposed to various doses of 25% ethanol administered by intraperitoneal injection, or varying durations of heat in a water-jacketed Thelco incubator on the morning of day 8. Doses of ethanol were 0.0075 ml/g body weight, 0.015 ml/g or two doses of 0.015 ml/g administered 4 h apart. Heat exposures were for 44, 50, or 56 min at 39.5° C. In order to test for possible interaction between heat and alcohol, animals were pretreated with 0.0075 ml/g 25% ethanol and then exposed to either 44, 50, or 56 min of dry heat on the morning of day 8. This dose of alcohol was chosen because, when given alone, it did not result in a significantly increased rate of fetal resorption or fetal malformation.

Blood alcohol levels at various times after injection of either 0.0075 or 0.015 ml/g 25% ethanol were determined on day 13. Blood samples were drawn by cardiac puncture just prior to

sacrifice. Samples were collected in heparinized venipuncture tubes. Blood alcohol levels were measured by the Clinical Laboratory of the Dartmouth-Hitchcock Medical Center, using the method developed by Calbiochem-Behring (Ethyl Alcohol STAT-PACK). Each sample was run in duplicate, and values showing significant variation between the two separate determinations were rejected.

Osmotic pumps (Alzet model 2001) were filled with 250  $\mu$ l of either a folic acid parenteral solution (Tradename Folvite, Lederle) containing 5 mg folic acid per ml or normal saline. Pumps were allowed to equilibrate in a saline solution for 4 h prior to being inserted subcutaneously on the afternoon of day 6. On activation, the pumps release their contents at a constant rate of 1  $\mu$ l/h. After anesthetizing each animal with sodium pentobarbital, a 1-cm midline incision was made through the skin on the back, and a single pump was inserted subcutaneously. The skin was closed with surgical clips. Forty hours later, on the morning of day 8, animals were treated with either two doses of 0.015 ml/g 25% ethanol or 50 min of heat. This time was chosen because it is the period of initial embryogenesis for neural tube closure in this species (9).

Blood was drawn for folate determinations via cardiac puncture on either day 8, 9, 12, or 13 after light ether anesthesia. Red cell folate levels were determined by radioassay in the laboratory of Dr. Brinck-Johnsen at the Dartmouth Medical School using the Corning Folate Radioassay kit (IMMO PHASE). Red cell folate levels were utilized because they are not subject to fluctuations due to daily folate intake and therefore provide more conclusive evidence of folate status. Samples were collected in glass tubes containing EDTA as an anticoagulant and frozen immediately after determining the hematocrit. Red cell folate levels were run in batches on duplicate paired samples and repeated if paired results differed by more than 15%.

Animals were sacrificed for fetal examination on the morning of day 13 and the number of sacs and resorption sites were recorded. All unresorbed fetuses were examined under a dissecting microscope for the presence of external anomalies, with particular attention to the head, limbs, back, and ribs (which could be visualized through translucent skin).

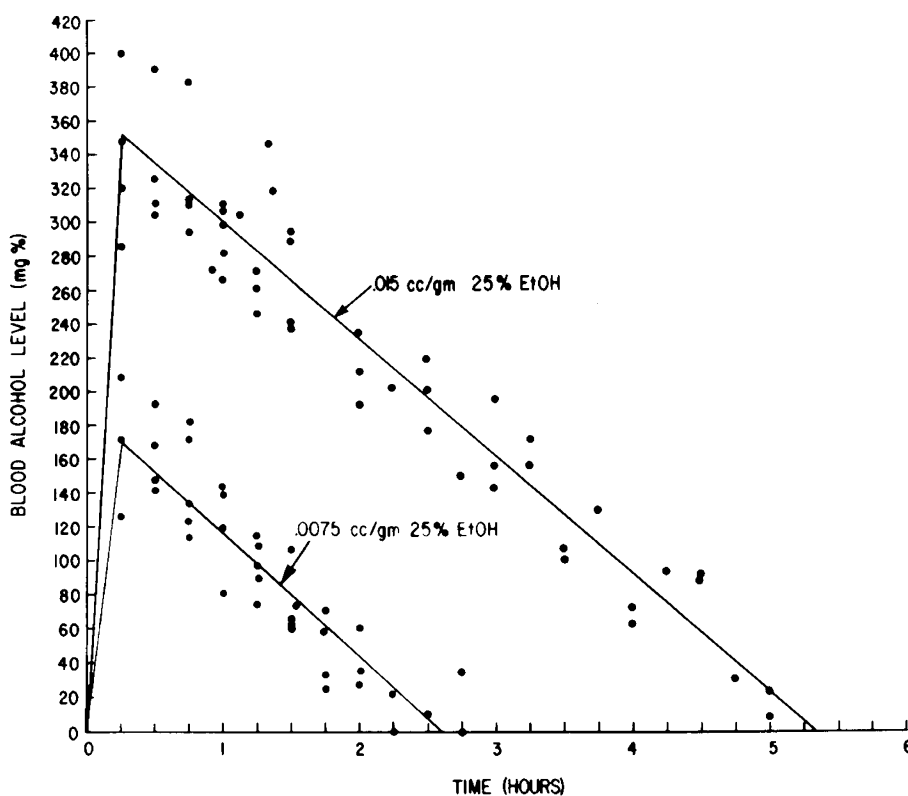


Fig. 1. Blood alcohol metabolic curves in the pregnant golden hamster.

## RESULTS

A single dose of 0.015 ml/g of 25% ethanol resulted in blood alcohol levels of about 350 mg/100 ml in 15 min (generally considered to be consistent with severe intoxication in the human); these levels gradually fell to 0 over the next 5 h (Fig. 1). Hamsters treated with this dose of alcohol appeared heavily sedated for the first 1 to 2 h, but were moving about with an unsteady gait within 2 h. Half of this dose (0.0075 ml/g) resulted in an average blood alcohol level of 175 mg/100 ml in 15 min, and these levels gradually fell to 0 by about 2½ h. Animals treated with this dose usually were moving about with an unsteady gait within 1 h. Exposure to 44, 50, or 56 min of heat was sufficient to elevate core body temperature 3 to 5° C as measured by a rectal temperature probe (YSI-TeleThermometer).

Except for saline pump controls, each treatment group included 10 to 15 litters. As shown in Table 1, there were no

Table 1. Rates of fetal resorption by treatment group

Treatment group	Total no. of litters	Total no. of sacs	% resorbed
Saline	15	187	5
0.0075 ml/g EtOH*	12	164	3
0.015 ml/g EtOH*	14	181	7
2 Doses 0.015 ml/g EtOH*	13	163	7
2 Doses 0.015 ml/g EtOH*†	13	200	5
2 Doses 0.015 ml/g EtOH*‡	7	100	5
44 mins heat	13	194	2
50 mins heat	12	163	10
56 min heat	13	182	27
50 min heat†	13	188	5
50 min heat‡	8	117	9
0.0075 ml/g EtOH* then 44 min heat	12	158	6
0.0075 ml/g EtOH* then 50 min heat	12	182	26
0.0075 ml/g EtOH* then 56 min heat	10	139	14

\* 25% ethyl alcohol.

† Folate pump inserted on day 6.

‡ Saline pump inserted on day 6.

significant differences in resorption rates with varying amounts of alcohol; however, increasing durations of heat exposure resulted in increased fetal resorption. Pretreatment with a half dose of ethanol just prior to heat exposure further increased the incidence of resorption, except at the 56-min duration when there was significantly higher maternal mortality associated with alcohol pretreatment. More than one-third of the mothers treated in that way failed to survive the treatment. With a 56-min heat exposure, there was a 35% maternal mortality following alcohol pretreatment, while heat alone for 56 min resulted in a 7% maternal mortality. The maternal mortality rate for a 50-min heat exposure after alcohol pretreatment was 24% versus 13% for heat alone at this duration. Heat exposure for 44 min did not result in any maternal deaths.

Dose-related trends were observed in the incidence of NTD with increasing amounts of alcohol; however, only the treatment with two doses of 0.015 ml/g ethanol 4 h apart reached statistical significance (Table 2). Figure 2 demonstrates differing outcomes observed in three fetuses from one litter exposed to two such doses of alcohol. Within the same litter, it was common to find both normal and abnormal fetuses. As shown in Table 2, the percent of NTD increased from 23 to 35 to 68% with increasing durations of heat ( $p = 0.03$  by F test). Pretreatment with a half dose of alcohol did not result in incidences of NTD that were significantly different from heat alone (Fig. 3).

Animals treated with low doses of 25% ethanol (either 0.0075 or 0.015 ml/g) did not demonstrate an increased incidence of NTD. The incidences of NTD in these animals were not significantly greater than zero (the rate observed in 15 saline-injected control litters and six uninjected control litters). Two doses of 0.015 ml/g 25% ethanol injected 4 h apart resulted in a significant increase in NTD ( $p < 0.01$  by Mann-Whitney U test).

As shown in Figure 4, animals with folate pumps had significantly higher red cell folate levels on day 8, and the levels continued to rise over the next 5 days. The incidence of NTD among folate-supplemented animals treated with either heat for 50 min or two doses of 0.015 ml/g ethanol 4 h apart were not significantly different by *t* test analysis from saline pump controls or similarly treated animals without pumps (Tables 1 and 2).

## DISCUSSION

NTD can be induced experimentally in laboratory animals by many different experimental insults during the time of neural

Table 2. Rates of CNS malformation by treatment group

Treatment group	Total no. of litters	Total no. of fetuses	No. of litters with NTD	No. of fetuses with NTD	% NTD	% fetuses with exencephaly
Saline	15	177	0	0	0	0
0.0075 ml/g EtOH*	12	158	1	1	1	0
0.015 ml/g EtOH*	14	169	2	4	2	1
2 Doses 0.015 ml/g EtOH*	13	151	12	67	44	28
2 Doses 0.015 ml/g EtOH*†	13	190	11	83	44	23
2 Doses 0.015 ml/g EtOH*‡	7	95	7	36	38	20
44 min heat	13	191	7	44	23	3
50 min heat	12	147	7	52	35	15
50 min heat	13	133	12	91	68	32
50 min heat†	13	178	11	87	49	13
50 min heat‡	8	107	7	48	46	8
0.0075 ml/g EtOH* then 44 min heat	12	149	6	33	22	3
0.0075 ml/g EtOH* then 50 min heat	12	135	11	45	33	14
0.0075 ml/g EtOH* then 56 min heat	10	114	10	87	73	44

\* 25% ethyl alcohol.

† Folate pump inserted on day 6.

‡ Saline pump inserted on day 6.



Fig. 2. Three 13-day hamster fetuses from one litter exposed to two doses of 0.015 mg/g body weight 25% intraperitoneal ethanol administered 4 h apart, on the morning of day 8. The fetus on the *left* demonstrates exencephaly, the *center* fetus has an encephalocele, and the fetus on the *right* is grossly normal.

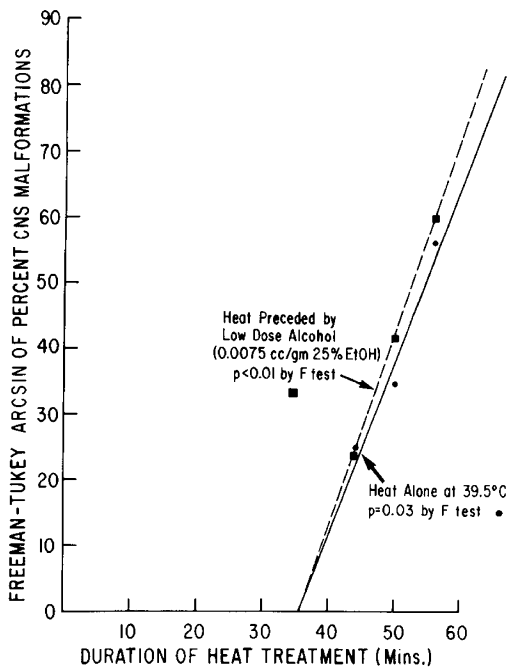


Fig. 3. Transformed percent of fetuses with NTD versus duration of maternal hyperthermia.

tube closure. Furthermore, the peripheral antifolic acid agent, Aminopterin, is reported to produce in NTD in man (32). For these reasons, our experiments were designed to determine what effect folic acid supplementation might have on experimentally induced NTD in a golden hamster model system.

The golden hamster is a well-accepted animal model system in experimental teratology (9). Golden hamsters are outbred, and, in more than 15,000 litters examined to date, Golden hamsters have been found to have less than a 0.1% spontaneous malformation rate. It is of note that NTD are not a spontaneous malformation in this animal, although they can be induced by a broad variety of teratogens administered on day 8 of gestation, which is the day of neural tube closure for this species.

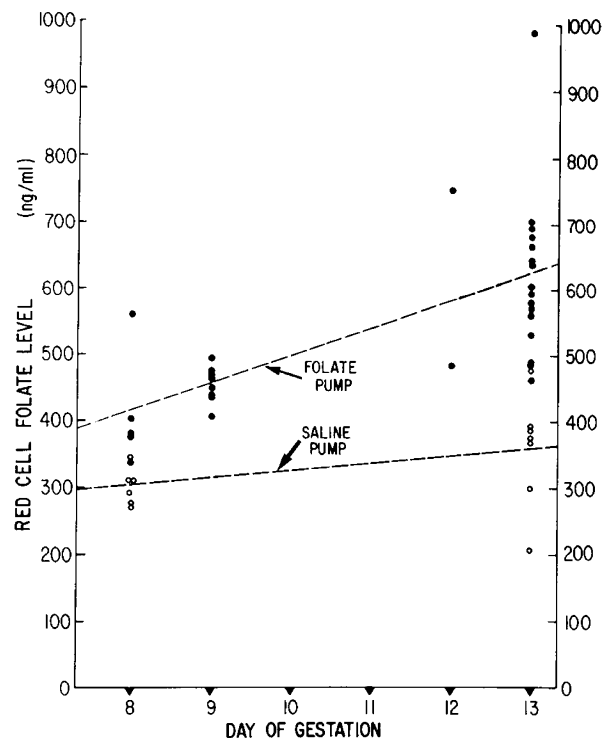


Fig. 4. Red cell folate level in pregnant golden hamsters versus day of gestation.

The studies described herein demonstrate that maternal hyperthermia and alcohol are both potent teratogens capable of inducing NTD in this model system. When mothers were supplemented with folate by means of an osmotic pump, no significant protection from NTD induced by either heat or alcohol could be demonstrated. Constant rate administration of folic acid by the osmotic minipump offers a consistent rate of exposure and results in stable maternal blood folate levels. Troughs and peaks of blood levels such as might occur with oral dosing are

avoided through this method of exposure, and there are no difficulties with noncompliance or poor absorption. The erythrocyte folate levels achieved by means of such osmotic pumps were quite comparable to those reported by Schorah *et al.* (24) among mothers receiving periconceptual vitamin supplementation and demonstrating a reduced NTD recurrence rate in comparison with a group of unsupplemented mothers (30).

Recently Smithells and coworkers (28, 30) reported a highly significant reduction in the recurrence rate for NTD among mothers given a periconceptual multivitamin supplement containing folate, when compared with the recurrence rate for unsupplemented mothers ascertained by the same centers over the same time period. It is generally accepted that NTD in man result from the interaction of genetic and environmental factors (1), and the widely observed social class gradient in the incidence of NTD has caused some investigators to suggest the possibility of a nutritional contribution to their causation. Smithells *et al.* (27) demonstrated significant social class differences in nutrient intake, and Laurence *et al.* (18) suggested a relationship between poor diet and NTD recurrence, with possible benefits resulting from dietary counseling.

In a prospective study of blood vitamin levels during the first trimester of pregnancy, mean blood levels of folate, ascorbic acid, and riboflavin were lower in a small group of mothers who later gave birth to infants with central nervous system malformations, than in controls. The authors noted that the upper limit of erythrocyte folate levels for women who subsequently had a fetus with a NTD was 170 ng/ml (29). When a subsequent group of women was treated with a preconceptional multivitamin supplement that contained a daily dose of 0.36 mg folic acid (given in three divided doses), mean erythrocyte folate levels rose from 250 ng/ml before supplementation, to 290 ng/ml after 28 days of supplementation, to 482 ng/ml by the 8th wk of pregnancy (24). By the 8th wk, all supplemented women had erythrocyte folate levels above the 170 ng/ml level for women who had had a NTD-affected pregnancy in the previous study (24, 29). When the data from this cohort (30) were combined with those from a subsequent cohort, the overall recurrence rates were 0.7% for 454 fully supplemented mothers and 4.7% for 519 unsupplemented mothers (28).

A double-blind, randomized control trial of folate supplementation alone was attempted in South Wales between 1969 and 1974, with 96 women who had previously had a child with a NTD (19). In this study, 4 mg of folic acid or a placebo was given each day, starting not later than the 1st day of the menstrual cycle during which a pregnancy was planned and taken through the end of the 3rd month. Unfortunately, 16% of the 60 women scheduled to receive folate failed to comply; there were two NTD recurrences among these noncompliers. There were four recurrences among the 51 women allocated to receive the placebo, and no recurrences among 44 women who complied with supplementation. The difference in recurrence rate among supplemented mothers *versus* unsupplemented mothers was significant at the  $p = 0.04$  level, when the two NTD recurrences born to noncompliers were added to the four NTD recurrences born to women in the placebo group.

Results reported herein suggest that in at least one experimental animal system, folate supplementation alone is not successful in preventing alcohol- or heat-induced NTD. It is possible that mothers who are successful in remembering to take a vitamin pill prior to conception and during early gestation may also be successful in remembering to avoid known teratogens. Such daily reminders of the importance of mothering from conception and thereafter could be extremely important. Further studies are needed in order to clarify the relationship between vitamin supplementation, particularly folate supplementation, and the reductions in NTD recurrence rates that are the subjects of current reports.

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