PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 187, 142–148 (1988)

Teratogenic Interaction of Ethanol and Hyperthermia in Mice (42647)

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Abstract. Alcohol and maternal hyperthermia have been implicated in human birth defects. Both ethanol and heat can induce neural tube defects (NTDs) and other developmental abnormalities in mice when large doses are given during pregnancy. To explore the teratogenic interaction of both agents, pregnant ICR mice were injected with a single dose of 25% ethanol and/or were heat-stressed in a water bath at 42°C on the morning of Day 8 of gestation. Combined treatment with ethanol (0.01–0.02 ml/g) and heat (10 min), when they were given concurrently or 1 hr apart, resulted in a significant increase of resorptions and externally malformed fetuses. Skeletal malformations and visceral variations also increased significantly following a concurrent exposure to both agents. These results indicate that ethanol and heat can be synergistically teratogenic in mice when the doses of each agent are below the teratogenic threshold. It was also suggested that pretreatment with a small dose of ethanol may not enhance the teratogenicity of heat when the hyperthermic stress is strong enough and teratogenic by itself.

Alcohol has been known as a human teratogen since the fetal alcohol syndrome (FAS) was delineated in 1973 (1). Since then, a number of epidemiologic and experimental studies have verified the range and degree of alcohol effects on the developing embryo and fetus. Malformations, intrauterine death, growth retardation, and behavioral dysfunction have all been recognized in humans and laboratory animals exposed to alcohol *in utero* (2, 3).

Maternal hyperthermia during pregnancy has also been implicated in human birth defects, with particularly damaging effects on the central nervous system (CNS) of the embryo (3, 4). Retrospective studies in humans have indicated an association between maternal fever episodes in early pregnancy and the occurrence of CNS defects in the offspring (5–11). Experimentally, neural tube defects (NTDs), microcephaly, microphthalmia and other defects have been produced in various animal species by heating pregnant animals (12–25). We have shown that a single heating of pregnant mice at 42°C for 12.5 min or at 43°C for 7.5 min can produce a significant number of offspring with NTDs and skeletal malformations, if the heat is applied at a critical period of teratogenesis (26).

Since developing embryos are highly vulnerable to the influence of alcohol and hyperthermia, and since pregnant women are at risk of being exposed to both agents concurrently, the present study was designed to examine whether heat and alcohol have a teratogenic interaction in mouse embryos, especially at relatively low exposure levels.

Materials and Methods. ICR strain mice (Shizuoka Laboratory Animal Center, Hamamatsu) were maintained in a temperature- and humidity- controlled animal facility with a 12:12-hr light:dark cycle. Animals were given laboratory chow and tap water *ad libitum*. Males and virgin females were housed together overnight, and the females were checked for the presence of vaginal plugs at 9:00 AM. The day on which a vaginal plug was found was taken as Day 0 of pregnancy. The females with vaginal plugs were randomly assigned to experimental groups.

Pregnant female mice were exposed to various doses of 25% ethanol administered by ip injection and/or varying durations of heat between 10:00 and 11:00 AM on Day 8, which is a critical period of neural tube closure. Hyperthermia was induced in mice by submerging the lower two-thirds of the body in hot water. The water bath was equipped

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with a thermoregulator (Taiyo Kagaku Kogyo, Tokyo) and a stirrer so that the water temperature could be maintained within a deviation of ±0.1°C from the set temperature. During the treatment, the animals were restrained in a plastic tube which was 3 cm in diameter and had several holes drilled in the sides. The dose of ethanol was 0.01, 0.015, or 0.02 ml/g body weight, and heat exposure was for 10 or 12.5 min at 42°C.

Each group was treated with ethanol and/or heat as shown in Table I. In order to explore the possible interaction between alcohol and heat, some animals were given ethanol immediately before heat stress (Groups VI–IX), and some were treated with both agents 1 hr apart (Groups X and XI). Control mice were injected with 0.02 ml/g distilled water and then exposed to 38°C for 12.5 min in a water bath (Group XII).

On Day 18 of pregnancy, all the females were killed by cervical dislocation. The uteri were removed and implantation sites, resorptions and dead fetuses were recorded. Live fetuses were sexed, weighed, and examined under a dissecting microscope for the presence of external anomalies. About half of the fetuses from Groups II, IV, VII, X, XI, and XII were fixed in 95% ethanol, cleared in 1% KOH, and stained with alizarin red S for skeletal examination (27). The remaining half of the fetuses from these groups were fixed in Bouin fluid and examined for internal soft tissue abnormalities by microdissection. Statistical analyses of maternal and fetal data were completed using the Student's *t* test for comparing means and the Wilcoxon rank sum test (28) for comparing distributions of precentages among litters.

The core body temperature during heat stress was monitored using nonpregnant female mice with and without ethanol pretreatment. Females weighing 32–36 g were restrained in a plastic tube and submerged in water at 42°C for 10 min, and their deep rectal temperature was monitored with a thermister thermometer (Shibaura Electric Co., Tokyo). The mice were wiped with soft paper towels when they were removed from the water in order to prevent hypothermia.

Blood alcohol levels were determined in nonpregnant female ICR mice at 15 min after the ip injection of 0.01, 0.015, or 0.02 ml/g 25% ethanol. Blood samples were taken by cardiac puncture and were collected in heparinized tubes. An aliquot of 0.5 ml of the blood sample was analyzed by gas chromatography by using the Shimadzu automatic head space analysis system for alcohol (GC-9A-HSS-2A) equipped with a flame ionization detector. The column was the Chromosorb 101, 80–100 mesh packed into a 3 mm i.d. × 210-cm glass tube. Blood alcohol concentration was calculated on the basis of peak area ratios of alcohol to 1.0 mg/ml of isopropanol as an internal standard.

Results. The results of the teratologic evaluation of term fetuses are presented in Table I. Generally, dose-related trends were observed in the incidence of resorptions and malformed fetuses with increasing amounts of alcohol and increasing durations of heat stress, when they were given alone. Animals treated with 0.01 or 0.015 ml/g alcohol (Groups I and II) did not demonstrate an increased incidence of externally malformed fetuses or resorptions as compared with controls. The incidence of malformed fetuses (1/106; 0.9%) from the females heat-stressed for 10 min (Group IV) was not significantly different from zero.

Heating for 10 min just after injecting alcohol resulted in an increase of malformed fetuses. The heat stress for 10 min following the pretreatment with 0.015 or 0.02 ml/g ethanol (Groups VII and VIII) increased malformed fetuses to 12.2 and 24.4%, respectively, which were significantly higher than the malformation rates in animals treated with ethanol or heat alone ($P < 0.01$). The malformations induced were mostly anterior neural tube defects such as anencephaly and exencephaly (Fig. 1a). Cleft palate and open eyelids were sometimes associated with NTDs. The incidence of resorptions increased significantly when the heat exposure was combined with 0.01 or 0.02 ml/g alcohol (Groups VI and VIII). Most deaths had occurred early in the embryonic stage, which corresponds to the period soon after the treatments. With a 0.02 ml/g alcohol injection, there was a 50% maternal mortality during the posttreatment with heat.

The prevalence of skeletal malformations and visceral variations increased significantly by concurrent treatment with ethanol and heat (Table II). The skeletal malformations induced were mostly of the axial skele-

TABLE I. TERATOGENIC EFFECTS OF ETHANOL AND HYPERTHERMIA AND THEIR INTERACTION IN MICE

Group	Treatment	No. of pregnant animals	% Dead mothers	No. of implants	% Resorption	% Survivors externally malformed	% Survivors with neural tube defects
I	0.01 ml/g EtOH	9	0	104	10.6	0.0	0.0
II	0.015 ml/g EtOH	12	0	156	12.2	0.0	0.0
III	0.02 ml/g EtOH	9	0	107	19.6	2.3	2.3
IV	10 min heat	9	0	112	5.4	1.4	1.4
V	12.5 min heat	11	0	151	15.2	15.6	15.6
VI	0.01 ml/g EtOH, then 10 min heat	8	0	103	28.2*	5.4	5.4
VII	0.015 ml/g EtOH, then 10 min heat	14	0	187	12.3	12.2**	12.2*
VIII	0.02 ml/g EtOH, then 10 min heat	12	50	70	44.3*	24.4**	22.0**
IX	0.015 ml/g EtOH, then 12.5 min heat	14	14	163	5.5	7.8	7.8
X	0.015 ml/g EtOH and 10 min heat in 1 hr	9	0	134	19.4	8.3*	8.3*
XI	10 min heat and 0.015 ml/g EtOH in 1 hr	12	0	172	24.4	8.5*	5.4
XII	Control	9	0	111	14.4	0.0	0.0

*,** Significantly higher than the corresponding values for the treatments with ethanol or heat alone (*$P < 0.05$ and **$P < 0.01$ by Wilcoxon rank sum test).

ton, including fused ribs, waved ribs, and deficiency of ribs and vertebral bones (Figs. 1b and 1c). Visceral variations were incomplete or abnormal lobations of the lung. The average weight of live fetuses did not differ significantly among groups.

Combined treatments with alcohol and hyperthermia 1 hr apart also increased resorptions and malformed fetuses. The malformation rate increased to 8.3% when the mice were treated with alcohol first (Group X), and to 8.5% when they were heat-stressed first (Group XI). These prevalences were significantly higher than the malformation rates in the fetuses treated with either alcohol or heat alone, although they were lower than the corresponding value for the concurrent treatment with both agents (Group VII, 12.2%).

Hyperthermia itself was teratogenic when pregnant mice were heated for 12.5 min at 42°C (Group V). The incidence of malformed fetuses was 15.6%, which was significantly higher than the control value ($P < 0.05$). When females were heated for 12.5 min just after they were injected with 0.015 ml/g alcohol (Group IX), the malformation rate (7.8%) was lower than the rate obtained after heat-stress alone.

Figure 2 shows the deep rectal temperature of nonpregnant mice during and after heat stress for 10 min at 42°C. The deep temperature reached 42.0°C when they had been placed in water for approximately 6 min. After removal from water, their body temperature dropped to the normal level in about 5 min. If mice had been injected with ethanol prior to the heat stress, hyperthermia was prolonged and it took longer to cool the body as compared with the mice treated with heat alone.

Blood alcohol levels in nonpregnant females were 317 ± 4 (SD), 487 ± 24, and 696 ± 26 mg/100 ml at 15 min after the ip injec-

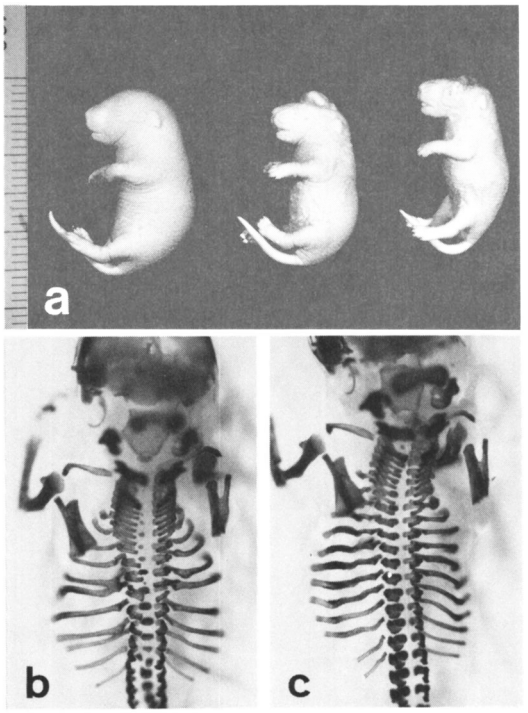

FIG. 1. Neural tube defects and skeletal malformations in term fetuses exposed to concurrent doses of 0.015 ml/g ethanol and 10 min heat at 42°C. (a) Exencephaly (middle) and anencephaly (right); normal fetus from a control mother (left). (b) Rib and vertebral malformations. Some ribs and vertebral arches are fused, and the number of rib pairs is smaller than normal by two. (c) Waved ribs.

tion of 0.01, 0.015, and 0.02 ml/g 25% ethanol, respectively.

Discussion. Embryotoxic and teratogenic effects of ethanol and hyperthermia have been investigated extensively using laboratory animals. As for the teratogenicity of alcohol, the mouse (29–36), rat (37, 38), guinea pig (39), and pigtail monkey (40) are among the species being studied. In C57BL/6J mice, a single dose of 0.015 ml/g 25% ethanol was found to be slightly teratogenic on Days 7–10 of gestation (32). Sulik *et al.* (34) have developed a mouse model of human FAS by giving two doses (0.015 ml/g) of 25% ethanol on Day 7. Experimental studies have shown that heat can also produce malformations, particularly CNS defects (12–26). Both alcohol and hyperthermia are known to be embryolethal when large doses are given to pregnant animals. It should be noted, however, that both agents have the teratogenic threshold below which they produce no recognizable gross malformations.

The results of the present study have shown that relatively low doses of ethanol and heat can demonstrate a combined teratogenic action in mice; the administration of 0.015 or 0.02 ml/g of ethanol just prior to heat stress for 10 min at 42°C significantly increased the malformation rates. The effects were synergistic rather than additive.

As for the mechanisms underlying their

TABLE II. EFFECTS OF ETHANOL AND HYPERTHERMIA ON THE SKELETAL AND VISCERAL DEVELOPMENT IN MICE

		Skeletal examination			Visceral examination		
Group	Treatment	No. of fetuses examined	% Fetuses with skeletal malformations[a]	% Fetuses with skeletal variations[b]	No. of fetuses examined	% Fetuses with internal malformations[c]	% Fetuses with internal variations[d]
II	0.015 ml/g EtOH	70	0.0	75.7	64	0.0	4.7
IV	10 min heat	48	35.4	66.7	51	0.0	3.9
VII	0.015 ml/g EtOH, then 10 min heat	75	70.6*	76.0	73	1.4	23.3*
X	0.015 ml/g EtOH and 10 min heat in 1 hr	52	61.5*	63.5	54	1.9	14.8
XI	10 min heat and 0.015 ml/g EtOH in 1 hr	62	58.1*	77.4	59	0.0	11.9
XII	Control	46	0.0	19.6	49	0.0	10.2

[a] Fused ribs, waved ribs, and deficiency of ribs or vertebral bones.
[b] Asymmetrical or supernumerary sternebrae, split vertebral arches, and cervical or lumbar ribs.
[c] Dilated ventricles and severe hydronephrosis.
[d] Incomplete or abnormal lobations of the lung.
* Significantly higher than the values for Groups II and IV ($P < 0.05$ by Wilcoxon rank sum test).

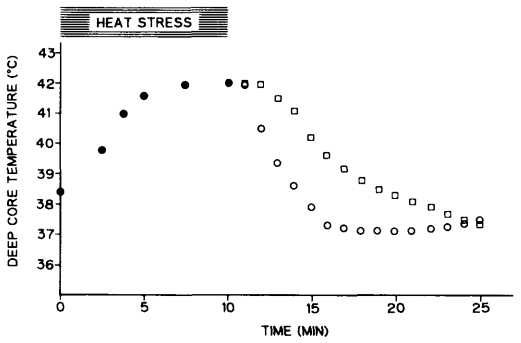

FIG. 2. The patterns of deep rectal temperature of female mice during and after heat stress for 10 min at 42°C. Solid circles show the deep rectal temperature during the heat stress and open symbols represent the temperature change after the mice were removed from water. Open circles, animals treated with hyperthermia alone; open squares, animals injected with 0.015 ml/g ethanol and then heated. Each point indicates the mean for three animals.

synergistic teratogenicity, one possible interpretation would be that ethanol pretreatment enhanced the teratogenic potential of heat by causing prolonged and sustained hyperthermia (Fig. 2). It was noted that the external and skeletal malformations produced in the present experiment were similar to those observed in mice exposed to hyperthermic stress *in utero* (21–26). Alcohol is known to cause vasodilation, especially of cutaneous vessels (41). Therefore, when mice are heated in water after alcohol injection, alcohol may enhance cutaneous blood flow and thereby an increased amount of heat may be incorporated into the body tissue to cause sustained hyperthermia. In the present experiment, blood alcohol levels elevated dose dependently in 15 min after ip injection, which may be sufficient to affect cutaneous vessels. Webster *et al.* (32, 36) injected 0.015 ml/g of 25% ethanol to C57BL/6J mice and found that their blood alcohol level was 350–400 mg/100 ml in 30 min. Thus, the blood alcohol levels in ICR mice appear to be not significantly different from the corresponding values in C57BL/6J mice.

It is also probable that alcohol and heat each affected embryonic development and their combined effects manifested as fetal abnormalities. This is supported by the fact that alcohol treatment in 1 hr after heat stress also increased the malformation rate significantly. If alcohol is given 1 hr after heating, the body temperature should return to normal level by the time of alcohol injection and therefore synergistically sustained hyperthermia cannot occur. Since ethanol passes easily into the embryonic compartment (42, 43), it is likely that the embryo of treated mothers is exposed to the concentrations of alcohol equivalent to those in maternal blood, which can be harmful to the embryo. In the mouse, Sulik *et al.* (34) noted extensive bleb formation in the neuroepithelium of the embryo after ethanol treatment. Prenatal exposure of rats to ethanol was shown to delay the proliferation of cortical neurons, reduce the number of neurons in the mature cortex, and alter the distribution of neurons (44). Brief hyperthermia can also cause a temporary cessation of cell proliferation, abnormal mitotic activity, and subsequent cell death in the neuroepithelium of heated embryos and fetuses (26, 45, 46). The developing neural tissue is so vulnerable to the teratogenic insult of ethanol and hyperthermia that their minimum toxic doses may synergistically result in fetal malformations.

Recently, it has been proposed that the heat-shock or stress response in mammalian embryos can alter the established developmental programs of gene activation/inactivation and result in abnormalities (47). Heat-shock genes have been shown to be inducible during development in lower animals such as *Xenopus* (48), *Drosophila* (49), and sea urchin (50). Walsh *et al.* (personal communication) treated explanted rat embryos with a nonteratogenic heat shock (42°C for 10 min) and detected a newly synthesized protein in the embryos. It has been reported that ethanol can also induce the heat-shock response (47). Thus, it may be possible that both hyperthermia and ethanol induce a heat-shock response that provides a common pathway leading to the production of developmental abnormalities. The hypothesis that heat-shock or stress response may be involved in teratogenesis is intriguing and awaits further investigation.

It is noteworthy that in the present study, pretreatment with alcohol did not increase the malformation rate or resorption rate when pregnant mice were heated for 12.5 min at 42°C, which is a teratogenic heat stress. Similar results have been reported by

Graham and Ferm (24), who heated pregnant golden hamsters after injecting ethanol. They heated the animals for 44, 50, or 56 min at 39.5°C in a water-jacketed incubator, and failed to obtain an increased incidence of NTDs as compared with the incidence obtained after the treatment with heat alone. It is interesting to note that their heating conditions were also teratogenic by themselves. Thus, pretreatment with nonteratogenic doses of alcohol may not enhance the teratogenicity of heat when the hyperthermic stress is strong enough and teratogenic by itself. Although we tried to examine the possible interaction between ethanol and longer heatings, all the mice pretreated with alcohol died when they were heated for 15 min or longer at 42°C.

It is widely accepted that alcohol is a human teratogen. Although the teratogenicity of hyperthermia is still a controversial issue (51), legitimate concerns have been raised regarding its potential hazards to human embryos. The present study has shown that alcohol and heat can be synergistically teratogenic in mice when the doses of each agent are below the teratogenic threshold. Although questions remain unanswered concerning the effects of such concurrent exposures in human embryos, it would be recommended that pregnant women should avoid alcohol consumption and unnecessary heat stress, especially in their early stage of gestation.

This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan and the Ministry of Health and Welfare of Japan.

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Received May 12, 1987. P.S.E.B.M. 1988, Vol. 187.
Accepted October 12, 1987.