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The Failure of Glutamic Acid to Protect the Rat Embryo Against the Action of Trypan Blue

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ABSTRACT The effect of L-glutamic acid on the embryolethal and teratogenic action of trypan blue was investigated in Wistar albino rats. L-glutamic acid was either incorporated into the diet, from gestation day 2 to day 20, or suspended in sesame oil and administered by gavage, from gestation day 6 to day 10. The day of finding sperm in the vaginal smear was designated day 0 of pregnancy. A teratogenic dose of trypan blue was injected at day 8 of pregnancy, either intraperitoneally (14 mg/kg maternal body weight) or subcutaneously (160 mg/kg). The amount of glutamic acid consumed, after the injection of trypan blue, ranged from 600 to 1,500 mg/rat/day. Pregnancy was terminated at day 20, and the fetuses were recovered and examined. Glutamic acid failed consistently to protect the rat embryo against the lethal and teratogenic action of trypan blue. These results are in contrast to those obtained in mice. The administration of sesame oil alone was found to cause embryonic death, but not malformations.

The disazo dye trypan blue has a long history as an experimental teratogen. It was first demonstrated to induce malformations in rats by Gillman et al. ('48), and was shown subsequently to produce malformations in a variety of animals (review by Beck and Lloyd, '66). Very little is known about the mechanism of action of trypan blue. A relationship between the yolk sac placenta and trypan blue teratogenesis was first postulated by Wilson et al. ('59). Trypan blue is avidly taken up by cells of the visceral endoderm of the rodent yolk sac and becomes localized in the supranuclear zone of these cells. Only very small amounts of the dye reach the embryo (Davis and Gunberg, '68; Davis and Sauter, '77; Dencker, '77). Beck et al. ('67) postulated that trypan blue interfered with the histiotrophic function of the yolk sac, and Williams et al. ('76) have demonstrated that trypan blue decreases the pinocytotic activity of rat yolk sac in vitro. Trypan blue also inhibits protein synthesis on free and membrane-bound ribosomes in the chorioallantoic placenta of mice (Tarachand and Eapen, '73).

Runner ('59) proposed that to be informative a teratological experiment should be reversible by an antidote. Such an experiment could be expected to give insight into the mechanism of action of the teratogen. Antimetabolites represent compounds that can be used in teratogen-antiteratogen experiments. There are only a few experiments, however, that report efforts to find an antiteratogen to trypan blue. In the chick, citric acid has been reported to decrease trypan blue-induced mortality and malformation (Beaudoin, '68), hyperoxia reportedly eliminates mortality (Hoffman and Ramm, '72), and hypothermia and hyperthermia decrease the incidence of induced tail defects (Kolesari and Kaplan, '74). Agarwal et al. ('60) reported that cortisone acetate offered complete protection against trypan blue-induced cardiac defects in rats. In the mouse, Zawoiski ('75) reported that L-glutamic acid significantly decreased trypan blue-induced resorptions and exencephaly, and L-valine decreased trypan blue-induced resorptions.

Exencephaly is the most frequently seen malformation in rats in my colony, following trypan blue treatment. It was decided, therefore, to test the reported protective effect of L-glutamic acid in these rats.

MATERIALS AND METHODS

Virgin female Wistar-derived rats from my colony were used. The animals were fed Teklad Rat Diet (Teklad Mills, Winfield, Iowa) ad libitum with weekly supplemental feedings of lettuce. The day of finding sperm in the vaginal smear was designated day 0 of pregnancy.

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In one series of experiments, L-glutamic acid (Sigma Chemical Co., Cleveland, Ohio) was mixed thoroughly with finely powdered rat chow at 1:20 or 1:12 and the mixture was fed from day 2 to day 20 of gestation. Food consumption was measured daily. In a second series of experiments, L-glutamic acid was suspended in sesame oil and administered by gavage from day 6 to day 10 of pregnancy. One-half the daily dose was given in 2 ml of sesame oil at 9 A.M. and the remainder was given at 3 P.M. of the same day. The total daily dose was 600 mg/animal. The gavage dose was selected to be the same dose (601 mg/day) used by Zawoiski ('75) in mice.

A teratogenic dose of trypan blue[1] was injected at gestation day 8, either intraperitoneally (ip) (14 mg/kg maternal body weight) or subcutaneously (sc) (160 mg/kg). Four types of controls were used: (1) trypan blue, (2) glutamic acid in the diet, (3) glutamic acid by gavage, and (4) sesame oil by gavage. Pregnancy was terminated at day 20. The resorption sites were counted and the fetuses were recovered, weighed, and fixed in Bouin's fluid for subsequent examination. Placentas also were examined and weighed.

RESULTS

Table 1 summarizes the results of this experiment. The administration of glutamic acid failed consistently to protect the embryo against the lethal and teratogenic action of trypan blue. In fact, the combined treatment produced a slight, although not statistically significant, increase in the incidence of resorptions and malformations over that following trypan blue treatment alone. There was no difference in the results when the glutamic acid concentration in the diet was increased from 1:20 to 1:12; therefore, these results are combined in Table 1. In the 20 animals receiving glutamic acid together with trypan blue, 91% (SD ± 11) of all implantation sites were affected adversely (resorbed or malformed). This compares with 76% (SD ± 20) of all implantation sites affected by trypan blue treatment alone. Because there was no evidence or suggestion of protection in any of the experiments performed, the use of additional animals was deemed unnecessary.

The results of this experiment suggest that sesame oil, in itself, may be embryolethal, but not teratogenic. The incidence of resorptions is similar when sesame oil is administered alone and when it is used as the vehicle for glutamic acid (Table 1). In both experiments, the number of resorptions is higher than the incidence (5.3%) of resorption seen in untreated controls in my colony during the past 20 years. A statistical comparison (χ^2) of the number of resorptions following sesame oil alone with the number of resorptions following glutamic acid alone (in diet) yields a P value of 0.04. The percentage of trypan blue-induced malformed fetuses in the present study (60%) is similar to the percentage of malformed fetuses observed in the cumulative data over the 20-year period (57%).

In this experiment, rats ate 22.6 ± 2.7 gm/day of powdered rat food containing either 1:20 or 1:12 glutamic acid prior to receiving trypan blue intraperitoneally. After injection, the rats consumed 20.4 ± 3.3 gm of food daily. The average intake of glutamic acid was 1.5 ± 0.32 gm/day prior to trypan blue injection and 1.3 ± 0.35 gm/day after injection. Rats receiving trypan blue subcutaneously ate 21.2 ± 4.0 gm/day of powdered rat chow containing 1:12 glutamic acid prior to the injection and 18.5 ± 3.1 gm/day following the injection. The corresponding amounts of glutamic acid ingested were 1.7 ± 0.36 gm/day and 1.5 ± 0.24 gm/day respectively. Trypan blue treatment resulted in a decrease in glutamic acid consumption of approximately 13%. The control rats receiving glutamic acid in their diet ate an average of 23.9 ± 3.3 gm of food per day throughout pregnancy with the resultant intake of 2.0 ± 0.26 gm glutamic acid daily. The amount of glutamic acid administered by gavage (600 mg/rat/day) was the same amount consumed by Zawoiski's mice ('75). In the present experiment, however, neither 600 mg/day of glutamic acid by gavage, nor 1,500 mg/day of glutamic acid consumed in the diet was able to protect the rat embryo against the lethal and teratogenic action of trypan blue.

Table 2 lists the types of malformations induced in this experiment. In spite of an apparent increase in frequency following the combined treatments, there are no statistically significant differences between the numbers of malformations induced by trypan blue alone and the number induced by trypan blue in combination with glutamic acid. Exencephaly was always the most frequently seen malformation, followed by absent eyelid and anophthalamia/microphthalamia.

[1] Trypan blue (Direct Blue 14) color index 23850, Lot B-430, Matheson, Coleman and Bell, Cincinnati, Ohio. This is from the same lot as the dye used by Zawoiski ('75).

DISCUSSION

The analysis of the results in this experiment revealed that there was a marked increase in the number of embryos dying following the use of sesame oil. The administration of sesame oil either alone or as the vehicle for glutamic acid resulted in an almost identical number of embryonic resorptions—16.2% and 15.6%, respectively. In a recent experiment with PBB, sesame oil was used as the vehicle for gavage administration (Beaudoin, '77). Nine pregnant rats received sesame oil by gavage at day 11, 12, and 13 of gestation. Of 109 implantation sites, 10% were resorbed

TABLE 1. The effect of L-glutamic acid on the embryolethal and teratogenic action of trypan blue

Treatment	Dosage	No. females treated	No. implantation sites	Resorbed (%)	Survivors malformed (%)	Fetal weight (gm) mean ± SD
Glutamic acid in diet plus trypan blue ip[1]	1 : 20 and 1 : 12, day 2–20 (1,300–1,500 mg/day) 14 mg/kg, day 8	6	91	63.7	75.7	3.20 ± 0.39
Glutamic acid in diet plus trypan blue sc[2]	1 : 12, day 2–20 (1,500 mg/day) 160 mg/kg, day 8	5	61	55.7	63.0	3.26 ± 0.28
Glutamic acid by gavage plus trypan blue ip	600 mg/day, day 6–10 14 mg/kg, day 8	4	42	61.9	93.7	3.15 ± 0.44
Glutamic acid by gavage plus trypan blue sc	600 mg/day, day 6–10 160 mg/kg, day 8	5	56	48.2	79.3	3.44 ± 0.18
Glutamic acid in diet	1 : 12, day 2–20	4	61	4.9	0	3.51 ± 0.02
Glutamic acid by gavage	600 mg/day, day 6–10	4	64	15.6	0	3.96 ± 0.02
Sesame Oil	4 ml/day, day 6–10	5	68	16.2	0	3.97 ± 0.04
Trypan blue ip	14 mg/kg	6	69	39.1	60.0	3.34 ± 0.38

[1] Intraperitoneally.
[2] Subcutaneously.

TABLE 2. Incidence of malformations following treatment with glutamic acid and trypan blue [1]

Treatment	Exencephaly	Anophthalmia/ microphthalmia	Absent eyelid	Hydrocephalus	Other[2]
Glutamic acid in diet plus trypan blue ip	76.0	36.0	56.0	4.0	16.0
Glutamic acid in diet plus trypan blue sc	70.5	35.3	47.0	5.9	17.6
Glutamic acid by gavage plus trypan blue ip	73.3	46.7	40.0	0	20.0
Glutamic acid by gavage plus trypan blue sc	87.0	17.4	56.5	4.3	8.7
Trypan blue ip	64.0	28.0	48.0	16.0	8.0

[1] Percentage of total number of survivors malformed.
[2] Encephalomeningocele and cranial blister.

and one fetus, from a mother treated at day 12, was found to be lacking the digits on all four paws. There was no effect on maternal, fetal, or placental weight. The spontaneous incidence of resorption in the rats used in my laboratory is 5.3%, based on 20 years cumulative data. A similar increase (10–25%) in resorption following the subcutaneous injection of sesame oil is reported by Palazzolo et al. ('72). The results of these experiments suggest that sesame oil can be lethal to the embryo during the early stages of rat embryogenesis. Further experimentation with other doses and other treatment days during gestation will be necessary in order to determine whether or not sesame oil also possesses teratogenic activity.

Zawoiski ('75) reported L-glutamic acid to be effective in reducing the number of trypan blue-induced resorptions and exencephalic fetuses in mice. L-glutamic acid was not effective as an antilethal or antiteratogenic agent in the present experiment. The reason why the results in the two experiments differ is not known. Each experiment contained animals injected with identical subcutaneous doses of trypan blue and receiving the same amount of glutamic acid. The present experiment, in addition, contained rats receiving intraperitoneal injections of trypan blue, gavage feedings of glutamic acid, and higher dosages in the diet than in the diet of the mice. Not one of the present experiments, however, gave any indication of a reduction in the number of trypan blue-induced resorptions or exencephalic fetuses. Actually a slight, although not statistically significant, increase in both resorptions and exencephalic fetuses occurred. Experiments with L-valine, reported to protect against trypan blue-induced resorptions (Zawoiski, '75), gave results similar to those of the experiments with L-glutamic acid. Combined treatment of pregnant rats with trypan blue and valine during the period of organogenesis resulted in the resorption of 67.9% of 262 implantation sites, and in malformations in 69.0% of the survivors: the majority with exencephaly (Beaudoin, unpublished).

Glutamic acid is a nonessential amino acid that can be made via transamination from αketoglutaric acid. In all species αketoglutarate is reported to be metabolically always present (White et al., '73). Glutamic acid is involved in many metabolic reactions, none of which suggests an obvious role in teratogenesis but several of which could be postulated to be involved in teratogenesis. Zawoiski ('77) concluded that the antiteratogenic activity of glutamic acid in mice was not due to its biotransformation into L-alanine, L-aspartic acid, or one of their metabolites.

The most apparent difference between Zawoiski's experiment and this experiment is the animal used (mouse and rat). There are differences in metabolic rates and in daily food consumption between the rat and the mouse, but what influence these differences may have on the outcome of the experiments is not known. It is accepted in teratology that species differences exist in the response to treatment with the same teratogen and at the same dose. The same dose of trypan blue, moreover, has been shown to induce different teratogenic responses in different strains of rats (Gunberg, '58). There are more similarities than dissimilarities between the present experiment and the experiment performed by Zawoiski. Glutamic acid metabolism is presumably the same in both the mouse and the rat (White et al., '73). Both animals are rodents with a yolk sac placenta, which is suspected of playing a role in trypan blue teratogenesis. The dye accumulates in the visceral endodermal cells of the yolk sac in both the rat (Wilson et al., '59) and the mouse (Greenhouse et al., '69), and the dye reduces acid phosphatase activity in these same cells in the rat (Beck et al., '67) and the mouse (Greenhouse et al., '69). Dencker ('77) has shown that trypan blue can be detected in the yolk sac fluid and in the wall of the embryonic gut in both rat and mouse embryos. The same dosage, route of administration, and time in gestation were used in each experiment, and similar malformations were induced by trypan blue in the rat and in the mouse fetus. The trypan blue used by Zawoiski and in the present experiment was not only from the same manufacturer, but from the same lot as well. At this time, no satisfactory explanation can be given for the discrepancy in the results.

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