Low Copper and Brain Abnormalities in Fetus from Triethylene Tetramine Dihydrochloride-Treated Pregnant Mouse

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Summary The effects of prenatal triethylene tetramine dihydrochloride (Trien-2HCl) exposure on fetal mice have been investigated on gestational day 19. Trien-2HCl was given throughout pregnancy at levels of 0 (control), 3,000, 6,000, or 12,000 ppm as drinking water, ad libitum. At the level of 12,000 ppm, the frequency of total resorption tended to be high and that of fetal viability tended to be low, as compared to controls. Decreased maternal weight was observed in body, but not in liver, at the level of 12,000 ppm. Fetal body and cerebrum weights significantly decreased at the levels of 6,000 and 12,000 ppm; however, fetal liver weight remained unchanged. Maternal serum copper concentration was not affected by the Trien-2HCl. Fetal copper concentrations of liver and cerebrum were significantly lower in the Trien-2HCl-treated groups than in the controls, with levels decreasing in a dose-related manner. When the copper and zinc concentrations in the group treated at 12,000 ppm were compared with those in controls, significant decreases in both metals were observed in placenta but not in maternal liver. Changes in fetal zinc concentration varied by tissues: i.e., an increase in liver and no change in cerebrum. Fetal abnormalities were frequently observed in brain, and the frequency was increased with increasing levels of the Trien-2HCl. These results suggest that fetal brain abnormalities caused by Trien-2HCl may be due in part to induction of copper deficiency, which is almost equivalent to that in brindled mutant mouse.

Key Words triethylene tetramine dihydrochloride, copper deficiency,

In this paper, the evidence of teratogenicity is understood as not only structural malformations but also functional deficits including organ weight such as a reduced cerebrum weight.
zinc, fetal mouse, pregnancy, brain abnormality, Menkes kinky-hair disease, Wilson’s disease, copper-chelating drug

Triethylene tetramine dihydrochloride [Trien-2HCl; 2,2’-ethylene diiminobis (ethylamine) dihydrochloride] is a chelating drug used in the treatment of Wilson’s disease, a disease in which excess copper is deposited genetically in the body (1, 2). Trien-2HCl is used primarily when patients show side effects to D-penicillamine (β, β-dimethyl-cysteine), the drug that was first prescribed for this disease (3). Recently, in Japan, Trien-2HCl has been known to be useful for Wilson’s disease (4). Several studies have, however, reported that Trien-2HCl is teratogenic in rats (5–8), but there have been only a few studies on mouse teratogenicity, and almost no information on brain in offspring.

The present study was designed to evaluate the fetal toxicity of Trien-2HCl and to determine its effects on metal concentrations in maternal and fetal tissues in mice. To the best of our knowledge, this report is the first to demonstrate fetal brain abnormalities resulting from maternal Trien-2HCl that are comparable to an animal model of Menkes kinky-hair disease (9), in which abnormal copper distribution is observed genetically in several tissues.

MATERIALS AND METHODS

Animals. The C3H/HeNjcl mice (Japan Clea Co., Tokyo) were given a standard diet (MM-3; Sankyo Lab., Tokyo) and tap water ad libitum. The MM-3 contained 10mg of copper/kg dry weight and any copper in tap water was below the detectable limit. Forty-five virgin females, mean 21 weeks of age, and 15 males were mated. Gestational day 0 was indicated by the presence of vaginal plug and sperm-positive vaginal smears. The pregnant mice were divided into four treatment groups: i.e., those given tap water containing Trien-2HCl (Tsumura Co., Tokyo, Lot No. 0024606030, 98.6% purity; diluted with tap water) at 0 (control), 3,000, 6,000, or 12,000 ppm, as drinking water.

Pregnancies were terminated on gestational day 19 at 10:00 by Cesarean section after chloroform anesthesia, except for spontaneous delivery by two dams. Maternal blood samples were collected by cardiac puncture. Dams of offspring were examined for body weight, litter size, gross abnormalities, and fetal viability. One hour after Cesarean section the live fetus was subjected to tissue weight determination, and biochemical or morphological analysis. Cerebral weight was obtained by weighing brain excluding the cerebellum and the lower brain stem.

Biochemical analyses. Metal analysis was conducted essentially as described in previous studies with brindled mouse (10–12). Frozen tissue was weighed and lyophilized. A lyophilized tissue was wet-ashed in HNO₃-HClO₄ and then the residue was dissolved in distilled water. Determination of copper, zinc, and magnesium was conducted by atomic absorption spectrometry.

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Copper was analyzed by graphite furnace atomic absorption spectrometry (Hitachi Model Z-8000 polarized Zeemann atomic absorption spectrometer equipped with an optical temperature controller). An uncoated graphite tube was used. Wavelength and slit width were 324.8 and 1.3 nm.

Zinc and magnesium were analyzed by air-acetylene flame atomic absorption spectrometry with one drop method (Hitachi Model 170-30 flame atomic absorption spectrometer equipped with a Teflon funnel for one drop method). Wavelength was 213.8 nm for zinc and 285.2 nm for magnesium.

The relative standard deviation \( (n = 10) \) was 2\% for each of the three metals. The detection limit \( (X_b + 3S_b) \) was 18 ppb for copper, 11 ppb for zinc, and 6 ppb for magnesium, where \( X_b \) is a mean value of blank and \( S_b \) is a SD of \( X_b \).

Protein was determined by the method of Lowry et al. (13).

**Morphological observation.** After examination for gross abnormalities and determination of body weight, any stillborn fetus was immersed in buffered neutral formalin solution. Two of three live fetuses per dam were randomly selected and removed heads were immersed in the same fixative. Only the ratios of fetus with external brain abnormalities are described in this paper; detailed morphological observation including microscopical finding is to be reported separately.

**Statistics.** Data were examined by Student's \( t \)-test or one-way analysis of variance (ANOVA), with Scheffé’s multiple comparison (ANOVA-MC) if necessary.

### RESULTS

**Fetal outcome**

Several parameters on fetal outcome are summarized in Table 1. The frequency of dams that experienced total resorption was increased when treated at 12,000 ppm. The total number of offspring per dam, including dead and live fetuses, did not differ among the four treatment groups. Fetal viability of litters which were kept at 25°C for 1 h after Cesarean section tended to be low in the 12,000 ppm group, as compared with the other three groups.

| Reproduction parameter | Trien-2HCl (ppm) |
|------------------------|------------------|
|                        | 0    | 3,000 | 6,000 | 12,000 |
| Dams with fetus per pregnant dams (%) | 13/13 (100) | 6/6 (100) | 10/11 (91) | 7/15 (47) |
| Number of dams | 13 | 6 | 10 | 7 |
| Total offspring per dam viability | 6.4±1.3 | 6.0±1.5 | 6.1±1.9 | 5.7±1.6 |
| Fetal viability 1 h after birth (%) | 94±10 | 94±9 | 92±13 | 76±24 |

Values are expressed as means \( \pm SD \) for the number of dams given.

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Maternal and fetal biological parameters

The effects of the four different treatment doses of Trien-2HCl on maternal and fetal biological parameters are shown in Tables 2 and 3.

In the maternal body weight on gestational day 19, a significant difference was detected only between the 0 and 12,000 ppm groups (Table 2). However, mean intakes of maternal diet or drinking water during pregnancy for 19 days did not show a significant difference between the 0 and 12,000 ppm groups (data not shown). Maternal liver weight also did not show any significant differences among the four groups (Table 2). In the placental weight, a significant difference was observed only between the 3,000 and 12,000 ppm groups (Table 2).

Fetal body weight was calculated for all live fetuses at birth. Mean fetal body

Table 2. Maternal body and liver weights, and placental weight on gestational day 19.

| Parameters     | 0            | 3,000        | 6,000        | 12,000       |
|----------------|--------------|--------------|--------------|--------------|
| Number of dams | 13           | 6            | 10           | 7            |
| Body (g)       | 35.5±3.4<sup>1</sup> | 34.4±2.6<sup>1</sup> | 34.6±2.6     | 31.3±2.9<sup>a</sup> |
| Liver (g)      | 1.73±0.23    | 1.66±0.10    | 1.65±0.15    | 1.61±0.16    |
| Placenta (mg)  | 87±6<sup>1</sup> | 97±9<sup>1</sup>    | 89±9         | 82±4<sup>b</sup> |

Values are expressed as means±SD for the number of dams given. <sup>1</sup>At Cesarean section, one dam in each of the 0 and 3,000 ppm Trien-2HCl-treated groups was already beginning spontaneous delivery; therefore, the number of one dam must be subtracted from the numbers given in body and placental weights. Placental weight is shown by mean values of all fetuses of a dam. The results of one-way ANOVA and then Scheffé analysis for multiple comparisons with α=0.05 are shown as follows: significantly different from <sup>a</sup>0 ppm, and <sup>b</sup>3,000 ppm.

Table 3. Fetal body, liver, and cerebrum weights on gestational day 19.

| Parameters     | 0            | 3,000        | 6,000        | 12,000       |
|----------------|--------------|--------------|--------------|--------------|
| Number of dams | 13           | 6            | 10           | 7            |
| Body (g)       | 1.28±0.10    | 1.24±0.10    | 1.17±0.07<sup>a</sup> | 1.08±0.07<sup>a,b</sup> |
| Liver (mg)     | 70±10        | 70±8         | 67±7         | 61±7         |
| Cerebrum (mg)  | 56±3         | 53±3         | 52±2<sup>1,a</sup> | 48±2<sup>a,h,c</sup> |

Values are expressed as means±SD for the number of dams given. Fetal body, liver, and cerebrum weights are shown by mean values of all observable fetuses of a dam. <sup>1</sup>Cerebrum from one dam was not available because of a decreased litter size; therefore, the number of one dam must be subtracted from the numbers given for cerebrum. The results of one-way ANOVA and then Scheffé analysis for multiple comparisons with α=0.05 are shown as follows: significantly different from <sup>a</sup>0 ppm, <sup>b</sup>3,000 ppm, and <sup>c</sup>6,000 ppm.

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Table 4. Copper concentration in maternal and fetal tissues of mice treated with Trien-2HCl at 3,000, 6,000, and 12,000 ppm.

| Tissue            | Trien-2HCl (ppm) |
|-------------------|------------------|
|                   | 0                | 3,000 | 6,000 | 12,000 |
| Number of dams    | 5                | 6     | 6     | 3      |
| Maternal serum    | 0.92±0.18        | 0.96±0.11 | 1.01±0.07 | 1.08±0.15 |
| Number of dams    | 10               | 5     | 6     | 7      |
| Fetal liver       | 89.0±17.6        | 54.0±7.7* | 50.3±7.5* | 33.5±9.4* |
| Fetal cerebrum    | 5.27±0.47        | 4.44±0.42* | 4.03±0.47* | 3.30±0.30*b,c |

Values are expressed as means±SD for the number of dams given (units: µg/ml in maternal serum, and µg/g dry weight in fetal liver and cerebrum). Copper concentrations in fetal liver and cerebrum are shown by mean values of two or three live fetuses of a dam. The results of one-way ANOVA and then Scheffe analysis for multiple comparisons with α=0.05 are shown as follows: significantly different from *0 ppm, b 3,000 ppm, and c 6,000 ppm.

weight was reduced in the 6,000 and 12,000 ppm Trien-2HCl groups (Table 3). However, there was no significant difference among the four groups in fetal liver weight which was calculated for all live fetuses (Table 3). Fetal cerebrum was weighed in the live fetuses at birth, except for those used for morphological examination. Mean fetal cerebrum weight was reduced in the treated groups in a dose-related manner (Table 3).

The data show that an adverse effect of Trien-2HCl on pregnant dam and on fetus is detectable from doses of 12,000 and 6,000 ppm, respectively.

**Maternal and fetal biochemical parameters**

Copper concentration in maternal serum, and in fetal liver and cerebrum in the four groups is shown in Table 4. Maternal serum copper level was not affected by Trien-2HCl under the experimental conditions. However, the serum copper was significantly lower in four dams (0.383±0.049 µg/ml; mean±SD) that experienced total resorption at the dose of 6,000 or 12,000 ppm. Copper concentration in fetal liver was dose-dependently decreased in the three treated groups as compared with the controls. Copper concentration in fetal cerebrum was also significantly decreased by Trien-2HCl treatment in a dose-related manner. Thus, copper concentration in fetus was affected by Trien-2HCl at the dose of 3,000 ppm and above.

**Concentrations of metals and protein**

To elucidate the mechanism underlying the adverse effect of Trien-2HCl on fetus, the copper, zinc, and magnesium concentrations and protein level in several maternal and fetal tissues were examined (Table 5). Data were obtained from the experiment wherein we paired mice receiving either 12,000 or 0 ppm from the beginning of mating of analysis of metals or protein. These samples were obtained
Table 5. Copper and zinc concentrations and protein level in maternal and fetal tissues of mice treated with Trien-2HCl at 12,000 ppm.

| Trien-2HCl (ppm) | Number of samples or fetuses | Maternal liver | Placenta | Fetal liver | Fetal cerebrum |
|------------------|-----------------------------|----------------|----------|------------|----------------|
| Cu 12,000        | 8                           | 13.1±1.1       | 7.19±0.75* | 31.5±14.1 | 3.29±0.53      |
| Zn 12,000        | 8                           | 123±5          | 122±8**   | 140±20**   | 104±3          |
| Protein 12,000   | 12                          | 125±4          | 107±4     | 265±62     | 104±5          |

Values are expressed as means±SD for the number of samples or fetuses given (units: µg/g dry weight in copper and zinc concentrations and mg/g wet weight in protein level). In each of the 0 and 12,000 ppm groups, four dams were used for these paired experiments: there were two samples per dam in maternal liver, and two (for Cu and Zn) or two to three (for protein) fetuses per dam in placenta, fetal liver, and fetal cerebrum. Significance between 0 and 12,000 ppm by Student's t-test: *p<0.01 and **p<0.001.

from the same experiments as shown in Table 4.

**Copper.** Maternal liver copper tended to be low in the 12,000 ppm (p<0.10) group as compared to 0 ppm. Copper concentration in placenta, fetal liver, and fetal cerebrum was significantly lower in the 12,000 ppm group than in the 0 ppm group.

**Zinc.** Maternal liver and placental zinc concentrations showed the same tendency as copper concentrations: i.e., Trien-2HCl treatment did not affect maternal liver zinc level but reduced the placental zinc. Contrary to the findings in copper concentrations, Trien-2HCl treatment significantly increased zinc concentrations in fetal liver but there were no changes in zinc concentrations in fetal cerebrum.

**Magnesium.** Magnesium concentrations in the four tissues consistently remained unaffected by Trien-2HCl treatment (data not shown).

**Protein.** Protein levels in fetal liver and cerebrum were not affected by Trien-2HCl.

These results demonstrated that change in copper and zinc concentrations varies with tissues: maternal liver having almost no changes, and placenta, fetal liver, and cerebrum having different zinc concentrations but consistently deficient copper concentrations. In fetal cerebrum, copper deficiency alone was observed, with no change in zinc concentration.

**Brain abnormalities**

There was an increase in the frequency of abnormal fetuses with increasing dose of Trien-2HCl (data not shown). Abnormalities were frequently observed in...
cranium or brain, including massive hemorrhages and hematomas, delayed ossification in cranium, exencephaly, microcephaly, and hydrocephaly. These brain abnormalities in live fetus were as follows (number of fetuses with abnormality per number of fetuses observed): 1.3% (1/79) in the 0 ppm group, 6.3% (2/32) in the 3,000 ppm group, 8.5% (5/59) in the 6,000 ppm group, and 39.0% (16/41) in the 12,000 ppm group. Regarding the quality of brain abnormalities, hemorrhages and delayed ossification were observed at the dose of 3,000 ppm and above, microcephaly and hydrocephaly were at the dose of 6,000 ppm and above, and then exencephaly was clearly observed at the dose of 12,000 ppm.

DISCUSSION

The present results on teratogenicity of Trien-2HCl in mice show that 1) orally administered Trien-2HCl induced much stronger adverse effects on fetus than its dam, 2) fetal tissues showed consistently low copper concentrations in a dose-related manner, despite variable levels in zinc concentrations, and 3) fetal biochemical and morphological abnormalities were clearly observed in brain. Walshe stated that Trien-2HCl has no teratogenic effects in mice, but no information on experimental design or data were provided (2). We have no other available report on teratogenic effects in mice. On the other hand, by using Sprague-Dawley rats, Keen and Cohen et al. conducted biochemical and morphological experiments on teratogenicity of Trien-4HCl in diet (5-8). However, they examined only whole fetus, liver and placenta as fetal tissues; there was thus no information on fetal brain or cerebrum. We believe that the present description on the teratogenicity of Trien-2HCl in fetal brain is the first such demonstration.

Although species and experimental design are different, similarities in teratogenic effects can be observed between rat studies (5-8) and the present mouse study. The methodological differences are as follows: 1) in rat study (6), Trien-4HCl was given as a diet, and 2) in our mouse study, Trien-2HCl was given as drinking water, throughout pregnancy. In both studies, with increasing amounts of Trien, the frequencies of both resorption and fetal abnormalities increased (5, 6, 8). Massive hemorrhages, edema, and reduced ossification observed in rat study (6) were also recognized in the present mouse fetuses, although our description is limited to only the head. Maternal and fetal weights show similar tendencies in both rat (6, 7) and the present mouse studies. Discrepancy is observed in maternal copper levels in serum or plasma and liver: 1) in the rat study, copper concentration in maternal plasma and liver was decreased by treatment (6, 7); however, 2) in the present mouse study, that in maternal serum and liver was not affected by the treatment. Fetal copper and zinc concentrations in the present study showed essentially similar tendencies by treatment to those in rat study (5-7). Furthermore, in both studies, magnesium concentrations were not affected by treatment in maternal and fetal tissues (6).

The mechanism leading to the embryo and/or fetal abnormalities caused by
Trien-2HCl is unknown. As a clue to resolve this problem, Trien-2HCl caused a decreased copper level but an increased zinc level in our fetal mouse liver, consistent with findings in fetal rat liver (5–7). Molecular localization of increased zinc in rat fetal liver after Trien treatment has been demonstrated in the metallothionein peak (8). On the other hand, D-penicillamine, a copper-chelating drug, caused decrease in liver copper and zinc concentrations in fetal rat (8, 14), in which cutis laxa, abdominal herniations, wavy and curved ribs, arthrogryposis, and spina bifida occulta were observed (14, 15). Differences in fetal liver zinc concentration and types of gross abnormalities between Trien and D-penicillamine treatments suggest that these two copper-chelating drugs may act in a different manner or at different molecular sites.

The low copper status in fetal cerebrum and placenta induced by Trien-2HCl in this study using C3H mice is comparable with that in brindled mice, C3H/HeJ-Mo<sup>br-l</sup>, as an animal model of Menkes kinky-hair disease, in which the primary defect is copper transport (9). In the placenta, 12,000 ppm Trien-2HCl caused a decreased in both copper and zinc concentrations; however, hemizygote mutant showed an increase in copper concentration and no change in zinc concentration (11). The mechanism underlying copper status in placenta was copper deficiency induced by Trien-2HCl and copper accumulation due to defective copper transport in brindled mutant, respectively. In the brain or cerebrum at perinatal stage, copper concentration was decreased under both conditions: 65% in 12,000 ppm Trien-2HCl and 49% (11) or 57% (16) in brindled hemizygote (mean% of control values), although zinc concentrations remained unchanged in both conditions. Furthermore, there is some evidence for beneficial effect of copper supplementation on fetus: copper supplementation to pregnant heterozygotes resulted in increased fetal viability of brindled hemizygotes (11, 12) and that to Trien-treated rat dams resulted in reduced number of fetal abnormalities (7). Therefore, Trien-2HCl-induced abnormalities in cerebrum may be explained in part by copper depletion alone at the almost same level as that in brindled mutant. The mechanism on the tissue-specific differences in fetal copper or zinc level induced by Trien-2HCl requires further study.

Regarding the management of pregnancy in Wilson’s disease which was treated with Trien-2HCl, Walshe has reported that there was no malformation in six infants (2), and further, that 11 pregnancies from seven patients with daily Trien-2HCl doses of 1.5–2.0 g resulted in nine live births including one child with chromosomal abnormality (there was one spontaneous miscarriage and one therapeutic termination) (17). Maternal serum copper or ceruloplasmin did not show a consistent value during pregnancy; however, the normal-for-age ceruloplasmin value was found in the cord blood (17). These data indicated that effect of Trien-2HCl on human fetus remains to be ascertained. In contrast with these data, our mouse study indicates that in a dose higher than 3,000 ppm Trien-2HCl, fetal low copper concentrations were detected in spite of no maternal abnormal findings. Roughly calculated by using weight of drinking water, the intake of Trien-2HCl at
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3,000 ppm level was approximately 500 mg/kg body weight/day in pregnant mice. Simple comparison of this dose of Trien-2HCl in our mouse with clinical treatment dose shows clear difference; however, for the extrapolation from mice to humans, we need further information on the metabolism of this drug in both humans and mice. Furthermore, as compared to pregnant mouse in our study, a pregnant human with Wilson’s disease may have a high concentration of tissues copper (140–340 μg copper/g dry weight in adult human liver of treated Wilson’s disease, unpublished observation, H. Tanaka); thus, there may be less-harmful effects of Trien-2HCl on fetal copper levels in humans than in mice. Finally we think this report suggests possible adverse effects of this drug on fetal brain function.

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