The Morphogenesis of Hindbrain Crowding Associated with Lumbosacral Myeloschisis

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Abstract

The teratogenicity of ethylenethiourea (ETU) was investigated in Sprague-Dawley rats and Landrace pigs. Pregnant rats each received a single intragastric dose of ETU on a given day from day 8 to day 19 of gestation, and pregnant pigs were given a single dose of ETU from day 15 to day 19. Control animals received an ETU-free vehicle. The newborn pigs were all normal, whereas there were high incidences of specific types of congenital malformation of the central nervous and other systems in the rats. A high incidence of lumbosacral myeloschisis associated with hindbrain crowding was observed in rat fetuses exposed to ETU on day 11 of gestation. This abnormality is considered comparable to Chiari type II malformation associated with spinal dysraphism (Arnold-Chiari malformation) in humans. Morphogenetic fetuses were examined by light and scanning electron microscopy and interactive image analysis. At gestational days 12 and 13, the volume of the caudal end of the neural tube from the anterior border of the hindlimb bud to the most caudal portion of the fetus was significantly greater in ETU-exposed than in control rat embryos. Experimental rat embryos also exhibited disturbance in closure of the posterior neuropore, with extrusion of neural tissue through the opening. An apparently unrelated abnormality in ETU-exposed rat fetuses was underdevelopment of the cranium, leading to hindbrain crowding.

The results of this study suggest that hindbrain crowding and lumbosacral myeloschisis in the rat are two separate phenomena, each of which occurs independently in response to an insult to the entire developing organism. Lumbosacral myeloschisis is caused by nervous tissue overgrowth that interferes with closure of the posterior neuropore, and hindbrain crowding is due to the growth of normal brain tissue in a too-small cranial cavity.

Key words: hindbrain crowding, myeloschisis, Chiari type II malformation, Arnold-Chiari malformation

Introduction

The combination of myelomeningocele and Chiari type II malformation is one of the most frequently encountered craniospinal dysraphisms in humans. Many attempts have been made to find a single explanation for these associated anomalies of the central nervous system (CNS). There are many theoretical mechanisms of these anomalies, including hydrodynamics, 2,6,10,24 developmental arrest, 5,11,12,17 traction, 12,21,32 overgrowth, 1,3,25,29,30,40 and primary para-axial mesodermal insufficiency. 22,23 However, a satisfactory pathomechanism has not yet been offered.

Hamburgh, 13 Gunberg, 12 Warkany et al., 40 and Rokos et al. 33,34 found that lumbosacral myeloschisis associated with hindbrain crowding could be induced in rat fetuses by administration of trypan blue to pregnant rats. Warkany and Takacs 39 and Warkany and O'Toole 38 observed similar results with administration of salicylate. These investigators contended that hindbrain crowding associated with lumbosacral myeloschisis is comparable to Chiari type II malformation associated with spinal dysraphism (Arnold-Chiari malformation) in humans. Therefore, investigation of the pathomechanism of Arnold-Chiari...
malformation in an appropriate experimental model would be not only of academic interest but also of clinical importance. However, the incidence of fetal anomalies induced by trypan blue and salicylate ranges from only 4.1% to 6.3%,33,34,38) which is too low to draw strong, meaningful conclusions.

Khera17) in 1973 and Sato et al.36) in 1985 reported that high incidences of specific types of congenital CNS malformations could be attained by the administration of ethylenethiourea (ETU) to pregnant Wistar17 and Long-Evans rats36) at specific gestational time points. The incidence of hindbrain crowding associated with lumbosacral myeloschisis in rats is 68.8%.17,36) However, there are differences within both species and strains in susceptibility to the teratogenic effects of any chemical.41) In rabbits17) and cats18) ETU administration has induced no malformations to date. Prior to our present study, it had not been determined whether or not ETU induces malformations in Sprague-Dawley (SD) rats or Landrace pigs similar to those observed in Long-Evans rat fetuses.

The present study was undertaken in an attempt to determine if morphological malformations of the CNS and/or other systems could be induced in SD rat and Landrace pig fetuses by administration of ETU. The morphogenesis of hindbrain crowding associated with lumbosacral myeloschisis in rats given ETU was also investigated by scanning electron microscopy, light microscopy, and interactive image analysis.

Materials and Methods

ETU (FBOML, Tokyo Kasei K.K.) was dissolved in distilled water at concentrations of 1% and 20% and used as a teratogenic agent in the experiments described below.

I. Experiment A-1

Mature, virgin SD rats weighing 220–240 gm and ranging in age from 10 to 12 weeks were used. Females were mated at night during the proestrus of the normal sexual cycle. The first day of gestation was considered to be the day when morning vaginal smears contained sperm.30) The pregnant rats were divided into 12 groups of nine each by gestational day (days 8 through 19). Each group was further divided into three subgroups and given ETU intragastrically through a gastric tube at a dosage of 60, 120, or 240 mg/kg (the acute oral LD₅₀ of ETU in the rat is 1832 mg/kg11). Each rat received a single dose of ETU on the assigned gestational day. The ETU-treated dams underwent Caesarean section on gestational day 20. The numbers of live and dead fetuses were recorded, and visible anomalies were examined under a dissecting microscope. A group of 12 control dams received ETU-free distilled water intragastrically and the same observations were made as in the experimental groups. The fetuses were fixed in Bouin’s solution for 2 months, then embedded in paraffin, and cut into serial sections 5 μm in thickness. The sections were stained with hematoxylin and eosin (HE) for microscopic study.

II. Experiment A-2

Mature Landrace female pigs 18–22 months old and weighing 80–90 kg were artificially inseminated. The day of insemination was considered the first day of gestation. Ten pregnant pigs were divided into five groups by gestational day (days 15 through 19). Each pig was given a single dose of ETU (240 mg/kg) intragastrically on the assigned gestational day. The ETU-treated dams delivered naturally at full term. The motility and external morphological features of the fetuses were examined.

III. Experiment B-1

Each of 20 pregnant rats was given intragastrical ETU, 240 mg/kg, on day 11 of gestation. Five ETU-treated dams were sacrificed by ether overdose every 12 hours, from gestational day 11.5 through 13. The uterine horns were removed and placed in physiological saline. The embryos were rapidly dissected from uterine and extraembryonic tissue under a dissecting microscope. A control of group of eight pregnant rats received an equal volume of distilled water and underwent the same procedures as the experimental animals. All the embryos were immersed in 0.02 M phosphate buffer at pH 7.2 for 2 hours and then fixed with a solution of buffered 2% glutaraldehyde containing 0.01% trinitrophenol16) at room temperature for 24 hours. The specimens were further dehydrated in a graded ethanol series, then washed with isoamyl acetate and absolute ethanol (1:1), and finally with absolute isoamyl acetate. The samples were dried in a critical point dryer (Ladd 28,000) and immediately sputter-coated with pure gold to a thickness of about 200 Å and examined under a Bausch and Lomb Model Nanolab-2100 scanning electron microscope at 15–20 kV.

IV. Experiment B-2

Three 12- and 13-day-old normal and experimental rat embryos were fixed in Bouin’s solution for 2 weeks, then embedded in paraffin and serially cut into 5-μm coronal sections and stained with HE. The total volume of the caudal end of the neural tube...
from the anterior border of the hindlimb bud to the most caudal portion of the posterior neuropore was calculated as the area of each section of neural tube, as measured by interactive image analysis (IBAS-I, Carl Zeiss), multiplied by the thickness of each section.

V. Experiment B-3
Ten 20-day-old control and experimental rat fetuses were fixed in Bouin's solution for 2 months, then embedded in paraffin and cut serially into sagittal sections 5 μm in thickness. The sections were stained with HE, and the mean area of the brain and cranium in midsagittal sections was measured by interactive image analysis (IBAS-I, Carl Zeiss).

VI. Experiment B-4
The angle of pontine flexure of 17- and 20-day-old normal fetal rats, and that of 17- and 20-day-old fetuses that had been subjected to a single dose of ETU on day 11 of gestation were examined in midsagittal sections.

All values obtained from experiments B-2 through B-4 are expressed as means ± SD. The significance of differences between control and experimental groups was determined by Student's t test.

Results

I. Experiment A-1
The pregnant dams given ETU neither died nor manifested signs of acute toxicity at any dosage. A total of 717 fetuses were obtained from 108 dams by Caesarean section on day 20 of gestation. High fetal mortality was noted in dams given ETU on gestational days 8, 9, and 10, the dosages having exceeded the teratogenic zone at this stage; the mortality rate steadily declined thereafter and remained at 4.3% (Fig. 1). In all fetuses obtained from dams treated with ETU on gestational days 11 through 18, there were not only external anomalies involving the central nervous system, but also anomalies of other systems (Table 1). Malformations were not observed in fetuses of control dams. The histological features of CNS malformation in the experimental fetuses were the same as described in our previous report.

II. Experiment A-2
Malformations were not observed in full-term newborn pigs. There were no differences in appearance or activity between the control and ETU-exposed piglets during the 8-week postnatal observation period.

III. Experiment B-1
In control rat embryos, the anterior neuropore closed on day 12 of gestation and the posterior neuropore on day 12.5, as is normal. In ETU-treated rat embryos, the anterior neuropore closed normally on day 12. However, closure of the posterior neuropore was disturbed. The neural tissue at the neural fold in the posterior neuropore showed overgrowth and bulging 12 hours after ETU administration. Finally, the hypertrophic neural tissue protruded outward over the ectoderm to form a myeloschisis (Fig. 2).

IV. Experiment B-2
The volume of the caudal end of the neural tube from the anterior border of the hindlimb bud to the most caudal portion of the posterior neuropore in three 12-day-old control rat embryos was 0.0106 ± 0.007 mm³ (Fig. 2C), while that of three myeloschistic rat embryos of the same gestational age was 0.0134 ± 0.0016 mm³ (Fig. 2G). This difference is statistically significant (p < 0.05). The volume in three 13-day-old control embryos was 0.0246 ± 0.0033 mm³ (Fig. 2E), and that in three myeloschistic embryos of the same gestational age was 0.0384 ± 0.0041 mm³ (Fig. 2I). This difference is also statistically significant (p < 0.05).

V. Experiment B-3
Midsagittal sections of the heads of experimental 20-day-old rat fetuses demonstrated crowding in the posterior cranial fossa. The choroid plexus of the fourth ventricle and the caudal portion of the cerebellum and medulla oblongata had herniated through the foramen magnum into the upper cervical spinal canal. There was no hydrocephalus (Fig. 3). The mean areas of the cranium in midsagittal sec-
tions of 20-day-old control and experimental fetuses were 30.16 ± 1.20 mm² and 27.22 ± 1.49 mm², respectively (Fig. 3C, D). This difference is statistically significant (p < 0.01). The mean areas of midsagittal sections of brain of control and experimental rat fetuses were 22.55 ± 1.01 mm² and 23.51 ± 1.60 mm² on day 20 of gestation, respectively. This difference is not statistically significant.

V. Experiment B-4

The mean angles of pontine flexure in 17-day-old control and experimental rat fetuses differed significantly (79.9 ± 0.46 and 92.3 ± 0.46 degrees, respectively; p < 0.01) (Fig. 4). In 20-day-old control and ETU-exposed fetuses the difference was also significant (124.3 ± 0.72 and 102.6 ± 0.26 degrees, respectively; p < 0.01) (Fig. 3C, D). Thus, in comparison with experimental rats, the angle of pontine flexure in controls was smaller at 17 days of gestation but larger at 20 days.

VI. Experiment B-4

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Discussion

The results of this study demonstrate that a single intragastric dose of 60, 120, or 240 mg/kg of ETU in pregnant SD rats from days 11 through 18 of gestation is capable of inducing fetal CNS malformations identical to those observed in Long-Evans rats. These include lumbosacral myeloschisis associated with hindbrain crowding, exencephaly, encephalocele, microcephaly, and degenerative hydrocephalus. Non-CNS malformations, such as shortness or absence of the tail, syndactyly, hypognathus, omphalocele, and anal atresia, were also seen. The occurrence of these malformations depends on the developmental stage at which the teratogenic agent is given. It appears that each organ or system has a specific period of susceptibility early in the development of its primordium.14,19,20) In this study, the failure to induce malformations in pigs may have been due to species nonsusceptibility to ETU. Apparently, vulnerability to teratogens depends on genotype, since not only different species but also different strains and substrains react somewhat differently to the same agent.41)

The most significant finding in this study was the high incidence of lumbosacral myeloschisis associated with hindbrain crowding in the offspring of rats given ETU on day 11 of gestation. Pathologic examination showed this malformation to be comparable to Chiari type II malformation associated with myeloschisis (Arnold-Chiari malformation) in humans.12,38,40)

Two contrasting theories have been proposed to...

Table 1  ETU-induced congenital malformations of the central nervous and other systems in rat fetuses

<table>
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<th>Day of gestation (ETU; mg/kg)</th>
<th>No. of fetuses examined</th>
<th>Spinal dysraphism with hindbrain crowding (%)</th>
<th>Exencephaly (%)</th>
<th>Microencephaly (%)</th>
<th>Meningoencephalocele (%)</th>
<th>Hydranencephaly (%)</th>
<th>Hydrocephalus (%)</th>
<th>Tail anomaly (%)</th>
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*Total number of fetuses of dams subjected to intragastric administration of various doses of ETU.
explain the pathomechanism of spinal dysraphism; disturbance of the closure of the neural tube, and reopening of the neural tube after normal closure has taken place. The morphogenetic findings in rat embryos exposed to ETU on gestational day 11 and the data concerning volume of the caudal end of the neural tube in 12- and 13-day-old experimental and control fetuses in the present study support the overgrowth theory, i.e., that myeloschisis is the result of disturbed closure of the posterior neuropore due to overgrowth of neural tissue. However, while control rat fetuses and those with hindbrain crowding associated with myeloschisis had brains of almost equal size, the cranial cavities of fetuses with hindbrain crowding and myeloschisis were smaller than those of normal fetuses. This suggests that hindbrain crowding is not caused by brain overgrowth. Since we found no hydrocephalus or cerebral aqueduct stenosis in rats with lumbosacral myeloschisis associated with hindbrain crowding, we do not agree with the hydrodynamic theory, which states that Chiari type II malformation associated with spinal dysraphism is induced by increased intracranial pressure due to hydrocephalus. We noted reduction of the angle of pontine flexure in 20-day-old rat fetuses with hindbrain crowding, so arrested development of the pontine flexure resulting in downward
displacement of the hindbrain and cerebellum\textsuperscript{4,31} does not seem a plausible explanation. Mechanical traction from spinal dysraphism in the morphogenesis of Chiari type II malformation can also be ruled out because traction from lower spinal dysraphism would result in an increase in the angle of pontine flexure.

Demyer\textsuperscript{5} classified malformations of the CNS on the basis of the time of their appearance during three stages of morphogenesis: cytogenesis, histogenesis, and organogenesis. As mentioned above, susceptibility to a teratogen depends on the developmental stage at which the agent is administered. In two studies,\textsuperscript{35,41} malformation did not occur in mammals when the agent was applied prior to differentiation. Wilson\textsuperscript{41} explained this by emphasizing that all primitive cells are structurally and chemically alike and therefore would be expected to react in the same way. If the agent is potent enough to kill or severely damage the cells, all cells will tend to be thus affected and the embryo will die. If the agent inflicts only minor damage, the injured cells will be repaired. Thus, specific defects cannot occur until there are distinct groups of cells having different susceptibilities and potentials. In general, susceptibility to teratogens decreases as organ development advances, usually becoming negligible when the organ is substantially formed.\textsuperscript{14,41} After this point, the only effect may be inhibition of growth or, occasionally, pathologic degeneration.\textsuperscript{14}

CNS differentiation during the various developmental stages is different. In the rat, the anterior neuropore closes on day 12 of gestation and the posterior neuropore on day 12.5. The cranial portion of the neural tube differentiates earlier than the caudal portion, and the response to teratogens differs in these two areas. When ETU was administrated to pregnant rats on day 11 of gestation, the caudal part of the neural tube was damaged and then reconstituted through proliferation of cells that had escaped necrosis. Over-repair of the nervous tissue of the posterior neuropore led to overgrowth and extrusion, resulting in myeloschisis. Development of the cranial part of the neural tube was merely inhibited by ETU. In the present study, the angle of pontine flexure.
flexure in 17-day-old ETU-exposed rat fetuses was greater than that of controls of the same gestational age, and development of the cranial part of the neural tube was delayed.

These findings suggest that the presence of a small cranial cavity with an overfilled cerebrum and cerebellum is a prerequisite for the development of hindbrain crowding. The overabundant cerebral and cerebellar tissues forcibly reduce the angle of pontine flexure as they grow into the upper cervical spinal canal.23 The small cranial cavities and lordotic elevation of the basal chondrocranium in experimental rats suggest that the developing cranium was damaged by ETU, resulting in underdevelopment of the occipital bone, especially its basal component.

It should be pointed out that hypervitaminosis A and trypan blue are also known to induce early mesodermal alterations.19,20,22) The teratogen appears to have various targets within the rat embryo. Malformation of other systems that originate from mesoderm were found in this study as well, including syndactyly of both fore- and hindlimbs and omphaloecele.

In summary, the findings of this study show that hindbrain crowding is due to normal brain tissue growing within a small cranial cavity and that lumbosacral myeloschisis is caused by overgrowth of the nervous tissue, which interferes with proper closure of the posterior neuropore. These appear to be multifocal developmental abnormalities consequent to a single exposure of the fetal organism to a noxious agent.40

Acknowledgments

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