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Prenatal sodium arsenite affects early development of serotonergic neurons in the fetal rat brain

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ABSTRACT

Prenatal arsenite exposure has been associated with developmental disorders in children, including reduced IQ and language abnormalities. Animal experiments have also shown that exposure to arsenite during development induced developmental neurotoxicity after birth. However, the evidence is not enough, and the mechanism is poorly understood, especially on the exposure during early brain development. This study assessed effects of sodium (meta) arsenite shortly after exposure on early developing fetal rat brains.

Pregnant rats were administered 50 mg/L arsenite in their drinking water or 20 mg/kg arsenite orally using a gastric tube, on gestational days (GD) 9–15. Fetal brains were examined on GD16.

Pregnant rats administered 20 mg/kg arsenite showed reductions in maternal body weight gain and food consumption during treatment, but not with 50 mg/L arsenite. Arsenite did not affect fetal development, as determined by body weight, mortality and brain size. Arsenite also did not induce excessive cell death or affect neural cell division in any region of the fetal neuroepithelium. Thyrosine hydroxylase immunohistochemistry revealed no difference in the distribution of catecholaminergic neurons between fetuses of arsenite treated and control rats. However, reductions in the number of serotonin positive cells in the fetal median and dorsal raphe nuclei were observed following maternal treatment with 20 mg/kg arsenite. Image analysis showed that the serotonin positive areas decreased in all fetal mid- and hind-brain areas without altering distribution patterns. Maternal stress induced by arsenite toxicity did not alter fetal development. These results suggest that arsenite-induced neurodevelopmental toxicity involves defects in the early development of the serotonin nervous system.

1. Introduction

Inorganic arsenite, an abundant element in the earth's crust that naturally occurs in many minerals, has become an environmental pollutant due to its wide use in industry and agriculture. Arsenite contamination in the environment is generally regarded as causing significant health problems worldwide (Lim et al., 2014; Ng et al., 2003; Rai et al., 2010; Vahter, 2008).

In recent years, there has been heightened concern over the potential for occupational or environmental exposure of children and adolescents to arsenic. Clinical and epidemiological studies in several countries, including India and Bangladesh, China, and other countries in Southeast Asia, Central and South America, have shown that high arsenic exposure through tap water induced impairments in intellectual function, verbal abilities, long-term memory, and other cognitive deficits in schoolchildren (Hamadani et al., 2011; Rocha-Amador et al., 2007; Rosado et al., 2007; Tsai et al., 2003; von Ehrenstein et al., 2007; Wang et al., 2007; Wasserman et al., 2004, 2007). Thus, arsenite had detrimental effects on the development and function of the nervous system. Furthermore, effects of As on much earlier development of nervous system such as fetal brains are not clear.

Animal experiments have shown that arsenite can cross the placental barrier, reaching the conceptus after maternal exposure (Hood et al., 1987, 1988; Rodriguez et al., 2002; Xi et al., 2009). Moreover, maternal exposure to arsenic can result in reproductive and developmental toxicity in their fetuses, including retardation of fetal and offspring growth and increased fetal mortality and neural

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tube defects (Beaudoin, 1974; Hood et al., 1977). Abnormal behavior and impairment of learning and memory functions were also observed in rat offspring pre- and post-natally exposed to arsenite (Rodriguez et al., 2001, 2002; Xi et al., 2009). Although these studies assessed the ability of perinatal arsenite exposure to induce neurodevelopmental toxicities, there were gaps between the time of arsenite exposure and assessments of offspring. Little information, however, is available on the direct effects of arsenic (events shortly after exposure) following prenatal exposure in animals.

This study therefore assessed the adverse effects of prenatal arsenite on early rat fetal brain development. Since maternal exposure to arsenite may also affect fetal brain development through maternal stress due to arsenic toxicity, we also examined the effect of maternal stresses, such as food and physical restriction, on fetal brain development.

2. Materials and methods

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the Food and Drug Safety Center, Hatano Research Institute.

2.1. Animals and chemical exposure

Male and female Sprague-Dawley [Crl:CD (SD), SPF] rats were purchased from the Japan Charles River Laboratory (Atsugi, Japan) and housed in a room at the Animal Facility of Hatano Research Institute. Temperature and relative humidity were controlled, with a 12–12 h light–dark cycle (lights on at 07:00). The animals were fed standard rat chow and bottled water ad libitum. The rats were allowed to acclimate for at least one week after arrival. To obtain pregnant animals, virgin females were placed in a cage with a male (1–2 females/male) overnight. Females with sperm in their vaginas and/or vaginal plugs were regarded as pregnant, with the day of detecting sperm or plugs designated as gestation day (GD) 0.

Sodium (meta) arsenite ($NaAsO_2$, As) was purchased from Sigma-Aldrich (St. Louis, MO, USA). It was dissolved in water and administered immediately afterward.

Arsenic doses were based on previous studies and our preliminary results. Rodriguez et al. (2001) reported that locomotive activity was reduced and dopamine concentration in the midbrain increased when adult rats were orally administered 20 mg/kg As for 15 days. Exposure of pregnant rats to 50 mg/mL As in drinking water from GD6 until postnatal day 42 was found to affect learning and memory functions and neuromotor reflexes in their offspring (Xi et al., 2009). Embryolethal and teratogenic effects were observed when pregnant rats were injected intraperitoneally (i.p.), once per day, with 30 mg/kg As on GD7–12 (Beaudoin, 1974). Our preliminary results showed that pregnant rats orally administered 50 or 100 mg/kg (5 mL/kg) As on GD9 to15 resulted in soft stools, anemia, and piloerection on GD9, with most of these animals dying on GD10. All pregnant rats administered 50 mg/kg As i.p. or 100 mg/kg oral As on GD15 died the next day, indicating that As doses $\geq$50 mg/kg were close to the maximal tolerated dose, and were overdoses when administered repeatedly to pregnant rats.

Based on these results, pregnant rats were either given free access to drinking water containing 50 mg/L As ($n = 6$), or administered 20 mg/kg As orally with a gastric tube on GD9–15 ($n = 12$). The volume of the oral dose was adjusted to 5 mL/kg body weight based on each rat's body weight on GD9. Control rats were administered the water used to prepare the As solution ($n = 11$). Maternal body weight ranges on GD9 in each group were 279.5–420.8 g in the control, 276.7–355.3 g in the 20 mg/kg As group, and 278.6–406.6 g in the 50 mg/L As group, respectively.

Treatment period (GD9–15) is the period of main rat organogenesis. By GD16, the primordial brain structure has already been constructed. At this developmental stage, neural stem cells are still dividing in the neural epithelium layer, but begin to differentiate into immature neurons and migrate to developmental fate areas. Since basic morphological endpoints of rat fetal brains occur on GD16 (Kuwagata et al., 2009; Ogawa et al., 2005; Senuma et al., 2012), fetal brains after maternal As treatment were evaluated on GD16.

Maternal body weight and food consumption of each animal was measured on GDs 9 and 16. Water consumption was measured each day from GD0 to 16. The amounts of food and water were calculated before they were supplied to each cage and their remnants were measured with a feeder weight (g) or water bottle (g) next day to calculate the differences, which were regarded as daily food and water consumption (g/rat/day).

2.2. Immunohistochemistry of the fetal brain

At GD16 (11:00 $\pm$ 1 h), dams were deeply anesthetized with sevoflurane and subjected to Cesarean section. The numbers of implantations and dead fetuses were recorded. The fetuses were fixed in 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4, at 4 °C for 2 days, and weighed after fixation. Fetuses for examination were selected randomly. Fetal brains were embedded in 10% gelatin and coronal serial sections were cut to a thickness of 40 μm with a vibratome. Every third coronal section was collected in three bottles. To analyze the distribution of serotonin, all sections were collected in one bottle containing 0.01 M phosphate buffer, pH 7.4. One set of serial sections in each group was stained with cresyl violet (Nissl staining), whereas other sections were subjected to TUNEL staining (In Situ Cell Death Detection Kit, POD, Roche Molecular Biochemicals, Penzberg, Germany) and immunohistochemistry. For immunohistochemical staining, sections were incubated with a 1: 2000 dilution of rabbit polyclonal antibody to phosphorylated histone 3 (anti-phospho-histone H3 [pSer10]; Sigma), a 1:1000 dilution of sheep polyclonal antibody to tyrosine hydroxylase (TH; Pel-Freez, NA), or a 1:8000 dilution of polyclonal goat antibody to serotonin (5-HT; ImmunoStar, WI). After incubation with the appropriate secondary antibody, the sections were subjected to an avidin–biotin immunoperoxidase reaction using a Vectastain ABC kit (Vector Laboratories), with the antigen visualized using diaminobenzidine as substrate. Immunohistochemical staining was performed in a free-floating manner.

2.3. Fetal brain examination

Fetal brains were examined in Nissl stained specimens. Parameters measured included the lateral and longitudinal diameters of the cerebrum, the thickness of the cerebral cortex, the longitudinal diameters of the boundary areas of the fore- and mid-brains and the lateral diameter of the midbrain. All were measured using a microscope in the control and 20 mg/kg groups (Fig. 2).

The number of dead cells in the neuroepithelium of the fetal brain was estimated in Nissl-stained sections by a light microscope at 400× magnification. Twenty-seven brain areas were examined, including the frontal neocortex, central neocortex, caudal neocortex, striatum, septum, hippocampus, amygdala, anterior and posterior thalamus, anterior and posterior hypothalamus, mammillary body, pretectum, mesencephalic tegmentum, substantia nigra compacta and ventral tegmental gray, superior and inferior colliculus, central gray, isthmus, cerebellum and medulla. The distribution of phospho-H3-positive cells on the ventricular surface of the neuroepithelium was examined at 400× magnification in 17 areas of the fetal brain (frontal neocortex, central neocortex, caudal neocortex, striatum, septum, hippocampus, amygdale, anterior and posterior thalamus, anterior and posterior hypothalamus, mesencephalic tegmentum, substantia nigra compacta and ventral mesencephalic tegmentum, isthmus, cerebellum and medulla).

2.4. Content of As in maternal and fetal tissues

The concentrations of As in the maternal liver, the placenta, and in the fetus and fetal brain of control rats and those administered 20 mg/kg As were analyzed on GD16 using inductively coupled plasma mass spectrometry (ICP-MS). Since impaired brain development was observed only in rats given 20 mg/kg As, As concentrations were analyzed at this dose. The lower limits of As detection were 0.01 ppm in the maternal liver, placenta and fetus and 0.02 ppm, in the fetal brain.

2.5. Maternal stress experiment

During treatment with 20 mg/kg As, dams were considered to be under stress due to As poisoning, probably resulting in reductions in body weight gain and food consumption. Prenatal stress has been shown to influence the development of the fetal brain (van den Hove et al., 2011). To address the contribution of maternal stress to As-induced toxicity, dams were subjected to other types of maternal stress. On GD9–15, dams were restricted in a small mesh area for 2 h/day and fed with half the amount of food consumed by the control pregnant animals. The brains of seven fetuses from two litters were obtained on GD16 and stained with antibody to5-HT, as described above.

2.6. Distribution of serotonin neuron

To assess the distribution of 5-HT-positive cells in detail, each midbrain area was divided into eight regions, from the rostral to the caudal portions. Areas positive for 5-HT were measured in the median and dorsal raphe nuclei using Image J (ImageJ 1.45, NIH).

2.7. Data analysis

All data were analyzed by the Statistical Analysis Software Package, SAS®. Reproductive performance was compared for litters, whereas all other results were compared in individual animals.

The data were summarized as group means and standard deviations, and differences were analyzed by Student's t-test (parametric data) or the Aspin-Welch t-test (non-parametric data). A p value less than 0.05 was considered statistically significant.