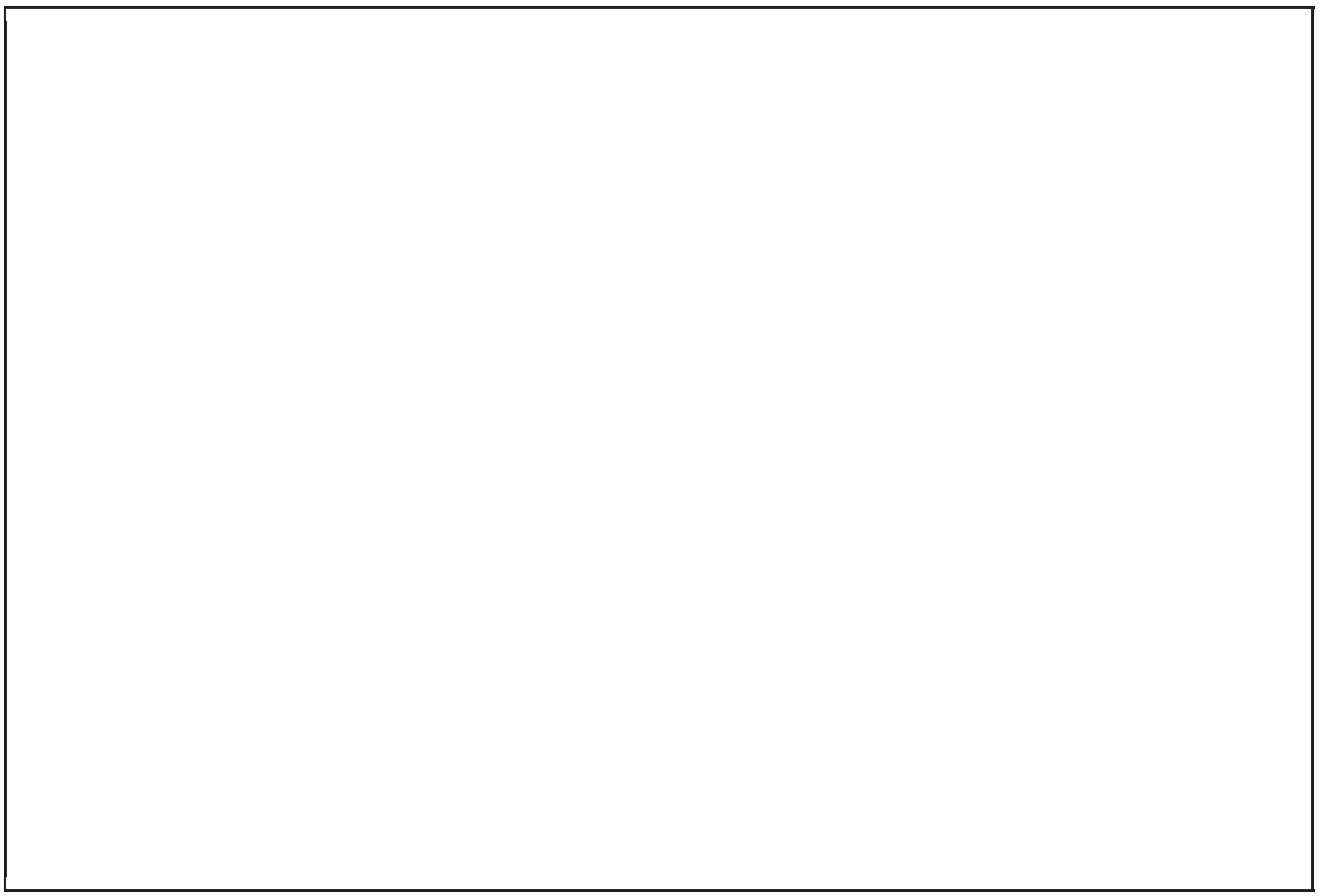
Effects of Low-Dose Drinking Water Arsenic on Mouse Fetal and Postnatal Growth and Development



placenta and cord blood [20,21,22,23]. Early life As exposure has also been associated with an increased incidence of cancer and bronchiectasis in adulthood, even several decades after cessation of exposure [24]. Interestingly, in Bangladesh, no effects have been observed on infant development at 7 or 18 months [25,26].

Based on cancer evidence, the U.S. EPA's current Maximum Contaminant Level (MCL) for As in public drinking water supplies is 10 ppb (0.13 μM) [27], which was recently revised from 50 ppb. Since that regulatory change, a number of studies have reported significant effects of As exposure at or below 10 ppb in experimental model systems [14,28,29,30]. The effects of low dose As at the current EPA standard on susceptible populations, such as pregnant women and infants, have been largely unexplored. Our goal was to develop a mouse model of early life As exposure through which we could identify critical windows of exposure that might result in adverse impacts on the development of the immune system later in life. However, following the development of our model, we unexpectedly observed that gestational and post natal As exposure at 10 ppb caused immediate effects on the rate of body weight gain of the F1 offspring. Thus, the studies presented here were designed to further explore the developmental outcomes following low dose As exposure during gestation and lactation.

Results

Birth outcomes

The experimental model of exposure is detailed in Figure 1 and the Methods section. Differences were not observed in any birth outcome (n = 14–17 dams per exposure), such as litter size (control: 7.5 (±0.3) pups; arsenic: 7.4 (±0.3) pups), gestational length (control: 20.2 (±0.33) days; arsenic: 19.6 (±0.38) days), the average weight of the litter or the survival of the pups (control: 1.28 (±0.01) grams; arsenic: 1.26 (±0.02) grams).

Growth of F1 offspring

Following birth, pups were monitored daily to assess survival and development. Survival and developmental milestones (eye opening, pinna unfolding, appearance of fur) was not differentially affected by the As exposure paradigm. As early as day 10 post natal, offspring exposed to As displayed significant decreases in growth (evidenced by total body weight), regardless of the timing of As exposure (Figure 2A). At the time of weaning (day 21 post natal) many of the As-exposed offspring were so small that it was not feasible to separate the offspring from the dams at the day of weaning. All pups were maintained with the dam until they reached a weight standard for separation of 7 grams. At weaning, mean weight values for IU, PN and IU&PN As exposed offspring were 7.5, 7.6 and 7.1 grams, respectively. The mean control weight was 9.2 grams. Given this cutoff for separation from the dam, percentages of mice requiring a delayed wean for control, IU, PN and IU&PN exposures were as follows: 0%, 33%, 25% and 46%, respectively. This growth deficit persisted through day 28 PN, regardless of whether or not a delayed wean was required. At day 42 PN, the growth deficit was still apparent in female but not in male mice (Figure 2B). Prior to this time point, both genders of mice were equally affected (Figure S1).

Arsenic tissue concentrations

To assess the direct exposure of the F1 offspring to As, total As levels in the placenta, dam's breast milk, pup stomach content (day 10 and 21 PN), and urine were measured by ICP-MS across all

opposing gestational exposure groups (control or gestational As) were exchanged and fostered. We observed that we could reverse the growth phenotype by simply exchanging the litters and dams from opposing exposure groups (Figure 6, Column C and D). Fostering within the exposure group recapitulated the original phenotype (Figure 6, Column E and F).

Discussion

In the conduct of an experiment designed to examine immune effects later in life from in utero exposure to As at the current U.S. EPA drinking water standard, we were surprised to observe significant short-term effects of these exposures on the F1 offspring and the dams. Specific effects included decreased growth of the F1 offspring and altered TG levels and profiles in the dams. Decreases in the nutrient content of the dam's breast milk, specifically TGs, appear to play a role in the growth deficits observed in the F1 offspring. The growth deficit was reversed by cross-fostering of the pups.

Arsenic concentrations were measured in the dam's milk, pup stomachs, placentas, and dam's urine, which confirmed that As was not transferred via the breast milk. In two exposure groups (IU and PN), we observed a trend towards decreased As levels in the milk, which was also recently reported in a similar mouse study with much higher doses of exposure [31]. These results are consistent with published reports of healthy lactating women exposed to As [8]. We did not detect significant increases in the levels of As in the placentas of the exposed mice compared to control mice. It is possible that As transfers through the placenta at a greater level in the exposed mice, but the low level of As used in this model and the detection limits of the ICP-MS impair our ability to measure it. Arsenic has been shown to transfer through

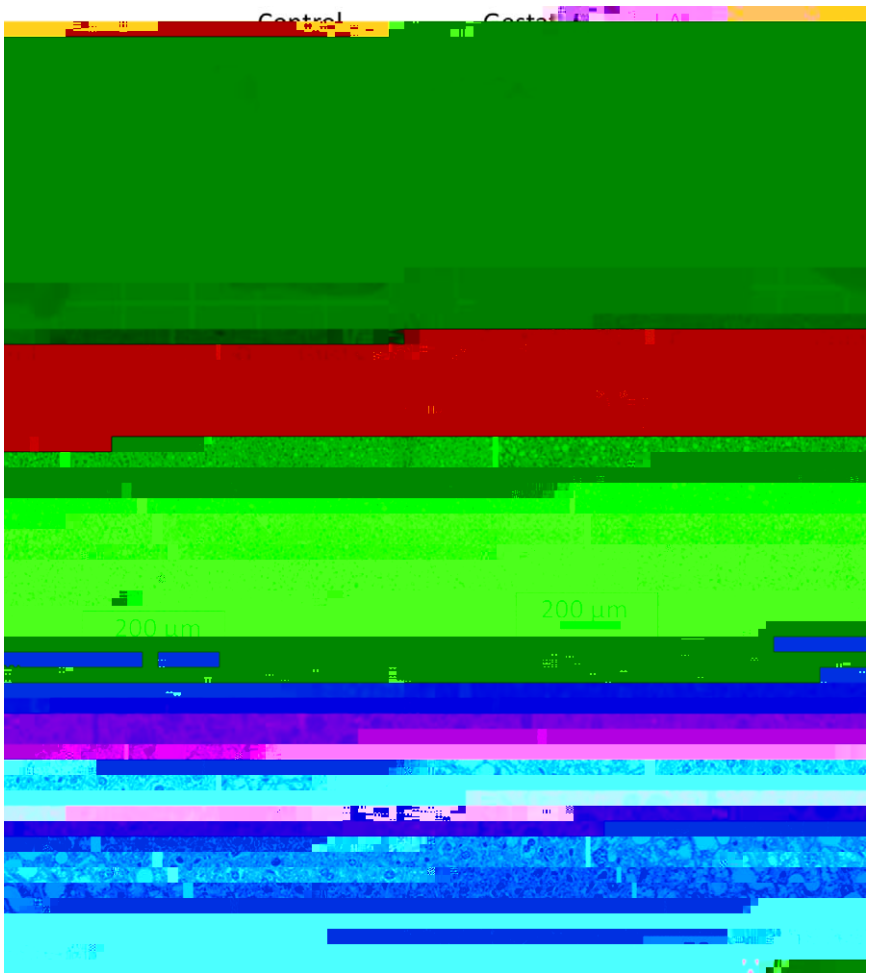


Figure 4. Effects of gestational arsenic exposure on liver steatosis and gross organ changes in the dam. Dams were sacrificed at gestation day 15.5. (A, B.) Detection of liver steatosis at the gross level was observed in a significant percentage of the As exposed mice (C–F.) Histological hematoxylin and eosin staining in (C.,E.) control and (D., F.) As-exposed dams. Scale bars indicate magnification.
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the placenta in human models of exposure and in mouse models with higher levels of exposure [6,7,32]. Given the dramatic effects on growth in the offspring, we were concerned that the pups may be acquiring exposure to As from another external source, such as the dam's urine. To assess such exposure, we also measured the stomach content of the pups. While we did not see a significant increase in the As levels in the milk, we did see a significant increase in the As levels the pup stomachs in two exposure groups:

Table 2. Exposure to Arsenic during gestation and lactation affect total TG levels in the serum and liver.

	Control	IU		
Gestational Day 15.5				
Serum TG (mg/dL)	119 (8.5)	74 (0.5)**		
Liver TG (mg/dL/mg liver)	4.1 (0.1)	6.85 (0.7)*		
	Control	IU	PN	IU & PN
PN day 15–18				
Serum TG (mg/dL)	110 (10.3)	70 (4.0)*	71 (9.9)*	65 (5.7)*
Liver TG (mg/dL/mg liver)	4.2 (0.6)	6.1 (1.4)	5.3 (0.7)	5.44 (0.4)

Asterisks indicate statistical significance,
*$p<0.05$ and
**$p<0.01$, compared to respective control. Values represent mean±SEM. Two tailed student's t-test for gestational exposure; One Way ANOVA for post natal exposures.
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IU and IU&PN at day 10 PN. However, it is unlikely that these elevated levels are a result of external exposure, via the urine or some other source, given that we did not observe such increases in the PN group. The dams in the PN exposure group excreted a significantly higher level of As in the urine compared to the IU group during the postnatal period. Thus, the basis for this increase in stomach As in these two groups is unclear. The overall levels of As in the stomachs at day 21 were increased compared to day 10 PN. This was likely a result of the pup's consumption of chow at this time point.

Interestingly, we also observed significant decreases in As excretion by the dams in all As exposure groups during the gestational and postnatal period, compared to virgin mice ingesting the same level of As. There was a trend towards increased water consumption and urine output in the As-exposed mice during pregnancy (and across all groups during the lactational period), but given the magnitude of these trends, the changes in water consumption and urine output are unlikely to account entirely for the significant decrease in urinary As output. Recent epidemiological data has shown that pregnancy can significantly alter the metabolism of As [33], but one challenge in the field has been extrapolating animal model studies of As exposure to human studies because mice are very efficient at metabolizing As [34]. Further research in this area is needed, but these data suggest that urinary As levels in pregnant mice may not be an accurate marker of the level of drinking water exposure.

The impeded growth of As-exposed offspring observed in this study was unexpected, given the low dose (10 ppb) of As used. Similar growth deficits have been observed in a high dose [10, 50 and 100 mg/L] IU exposure model of rats [35]. Other studies have used similar models of exposure (ppb and ppm levels of As during gestation and lactation), but have not reported changes in body weight gain early in life [10,14,32]. It has become clear in comparing different experiments that the dose of As is very important, as well as understanding and controlling the background concentrations of As in the diet and bedding [36]. Moreover, arsenic has been shown to display complicated, multiphasic dose response curves over a broad range that suggest different physiological effects at different dose ranges [37], so in retrospect it is not surprising to observe a different effect at these lower exposures, which might then be superseded by other effects at higher doses. We also recognize that many of these effects appear to be transient and can be experimentally ameliorated, suggesting that differences in the timing of exposure and in the experiment endpoints between this study and other studies using similar models could influence the observed results. It is also interesting that we did not observe greater effects in the groups with the combined exposure period (i.e., IU+PN) versus groups with only one or the other period of exposure (IU or PN). We cannot rule out the possibility that there is some capability to adapt to such low level exposures. Clearly, the response curves of As exposure, particularly at low levels, are complex, but these unexpected low-level effects on fetal growth warrant further investigation.

Based on our results, it is clear that As effects on the dam play a major role in the growth deficit of the offspring. The results of the fostering experiments suggest that the milk is the major contributor to the effects we have observed. However, we cannot

rearing of the pups did not play a role in the growth deficit. Breastfeeding of infants in human populations with As exposure has been shown to be protective against increased As exposure to the infant [38]. Our results suggest that As exposure can impact the composition and quality of breast milk, even when As is not being directly transferred via the breastmilk. We also observed an interesting gender-specific effect. These results suggest that female mice are more vulnerable to early life As exposure, compared to

the final solution was confirmed by induction-coupled plasma mass spectrometry (ICP-MS) metal analysis at Dartmouth's Trace Elements Analysis Core Facility. Drinking water was changed twice weekly.

Offspring growth

At birth, the gestational length, number of pups, average litter weight (weight of individual pups/number of pups in litter), number of dead pups, and number of pups with malformations were recorded. Weight (grams) was recorded for offspring at birth, day 10, day 21, day 28 and day 42 PN.

Liver histology

Liver steatosis in the dams was observed at the gross observational level. For histological confirmation, livers were removed and fixed in formaldehyde, paraffin embedded, sliced and stained with hematoxylin and eosin.

Collection of breast milk

Dams (day 10–12 postnatal) were separated from pups for 6 hours to allow for milk accumulation. Dams were lightly anesthetized (i.p.) with 9:1 ketamine:xylazine mix at 0.1 ml/30 g body weight and injected (i.p.) with 2 IU (100 uL) of oxytocin (Sigma-Aldrich, St. Louis, MO). Dams were milked by gentle manual stimulation of the teat and collection with a pipette. Milk was stored at −20 degrees C.

Analysis of protein levels

Milk samples were diluted in PBS and assayed for protein (1:400 dilution). Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL), according to manufacturer's instructions.

Collection of serum and livers

Dams were fasted for 6 hours. Blood was collected from the vena cava and livers were collected and snap frozen. Liver tissue (~150 mg) was homogenized in PBS using the Bullet Blender Homogenizer (Next Advance, Cambridge, MA).

Analysis of triglycerides

Triglyceride concentrations were determined in the dam's breast milk (1:160 dilution), liver tissue (1:20 dilution) and serum (1:2 dilution) samples from pregnant and lactating dams across all exposure groups, using Wako L-type triglyceride (Wako Diagnostics, Richmond, VA), according to manufacturer's instructions.

Lipoprotein profiling

Lipoproteins were separated using fast protein liquid chromatography (FPLC). 0.2 mL of pooled mouse plasma was injected onto a Superose 6B 10/30 column (GE Healthcare Life Sciences, Piscataway, NJ) and eluted at a constant flow of 0.2 mL/minute with phosphate buffered saline (pH 7.4). The effluent was collected in 0.2 mL fractions and triglyceride and cholesterol levels were determined for each fraction using L-type and Cholesterol E kits

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