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Developmental windows of susceptibility to inorganic arsenic: a survey of current toxicologic and epidemiologic data†

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Globally, millions of people are exposed to elevated levels of inorganic arsenic (iAs) *via* drinking water. Exposure to iAs is associated with a wide range of negative health outcomes, including cancers, skin lesions, neurological impairment, cardiovascular diseases, and an increased susceptibility to infection. Among those exposed to iAs, the developing fetus and young children represent particularly sensitive subpopulations. Specifically, it has been noted in animal models and human populations that prenatal and early life iAs exposures are associated with diseases occurring during childhood and later in life. Recent epidemiologic and toxicologic studies have also demonstrated that epigenetic alterations may play a key mechanistic role underlying many of the iAs-associated health outcomes, including the carcinogenic and immunologic effects of exposure. This review summarizes some of the key studies related to prenatal and early life iAs exposure and highlights the complexities in isolating the precise developmental windows of exposure associated with these health outcomes.

Introduction

Over 100 million people, worldwide, are estimated to be exposed to inorganic arsenic (iAs) *via* drinking water at levels that exceed the World Health Organization's (WHO) recommended limit of 10 ppb.[1,2] In addition to the massive poisoning occurring in Bangladesh, iAs has been identified at levels of concern in many other populations around the globe, including the United States.[2–5] Of public health concern,

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exposure to iAs at levels greater than 10 ppb has been associated with a wide range of negative health outcomes, including cancers of the skin, bladder, liver, and lung, immunological dysfunction, neurological impairment, increased mortality, and complications during pregnancy.[2]

There is growing evidence that the developing fetus and young children represent particularly sensitive subpopulations at risk of the harms of exposure to iAs through drinking water. Prenatal exposures to iAs are of concern because iAs crosses the placental barrier and is found in the umbilical cord at levels that are similar to those found in the mother.[6] Moreover, exposures during pregnancy are associated with diseases in offspring occurring during childhood and those emerging later in life.[7,8] There is additional evidence that early life exposures are similarly associated with later life disease.[9] Specifically, such exposures have been associated with cognitive impairment, increased susceptibility to infectious diseases, impaired pulmonary function, cardiovascular disease, and cancers occurring during adulthood.[10–14] In rural and developing regions where there is a reliance upon groundwater, rather than regulated utilities systems, pregnant women and young children are especially at risk for exposure to elevated drinking water iAs. With relevance to children's exposure, there is emerging evidence that rice products, which comprise a significant proportion of many children's diets, may represent a source of iAs exposure.[15–17] Therefore, it is critical that the risks associated with prenatal and early life iAs exposures are thoroughly understood.

In this review, we describe current epidemiologic and toxicologic data regarding health effects associated with prenatal and early life iAs exposure. Human studies have provided evidence that prenatal and early life exposures to iAs yield a range of adverse health outcomes including cancer and non-cancer endpoints. Here, we focus on a subset of these outcomes, including neurodevelopmental, reproductive, and immunological endpoints, along with evidence of the epigenetic underpinnings of iAs-induced disease. The studies reviewed here can be found in Tables S1 and S2. Importantly, in populations where children experience such exposures, the timing often occurs during the prenatal period, childhood, and adulthood. This impairs the ability to differentiate the effects associated with iAs exposure during specific developmental windows. Thus, this review highlights the importance of continued research, especially using animal models, to elucidate the important underpinnings of these complex health outcomes and to isolate the health effects associated with precise developmental windows of exposure.

Health effects of prenatal and early life exposure: epidemiologic evidence

There is great interest in understanding the health effects of prenatal and early life iAs exposure. Such exposures have been linked, in population studies, to pregnancy complications, adverse birth outcomes, epigenetic reprogramming, and cancer and non-cancer outcomes appearing both during childhood and adulthood.[7]

Much of the research on iAs exposure has been focused on its carcinogenic effects. Among the first human population-based evidence that prenatal and early life exposure to iAs was associated with cancers in both childhood and adulthood comes from ecologic studies in Antofagasta, Chile.[11,18,19] In Antofagasta, exposure to high levels of iAs (approximately 870 ppb) was limited to a defined time period, specifically 1958–1971, during which the city switched to an iAs-contaminated drinking water source. Given that extremely high iAs exposure occurred during a defined period of time and impacted all individuals throughout Antofagasta, this incident serves as a "natural experiment" in which the effects of iAs exposure during specific developmental periods can be studied. Utilizing mortality records from 1989–2000, researchers reported a significant increase in the mortality from bladder, laryngeal, lung, kidney, and liver cancers, along with chronic obstructive pulmonary disorders (COPD) and circulatory diseases in Antofagasta compared to other regions of Chile. Specifically, such an effect was noted among residents who were 30–49 years old and had experienced *in utero* and/or childhood exposure to the iAs-contaminated drinking water.[11] These findings have been critical in establishing the latent effects of prenatal and early life iAs exposure. While it is difficult to isolate *in utero* from early life exposure, these results set the stage for continuing research into these latent effects.

Evidence that early life exposures could be associated with other latent and persistent effects occurred following the Morinaga dried milk poisoning in Japan in the 1950s. In this incident, infant formula was contaminated with approximately 4000–7000 ppb of iAs, corresponding to a daily dose of over 500 μg per kg body weight.[20] During the poisoning, high numbers of infants suffering symptoms of acute iAs toxicity were reported and more than a hundred infants died. However, among those that survived, there is evidence suggesting that iAs exposure is associated with a range of neurological effects, including epilepsy, lower Intelligence Quotients (IQ), and higher rates of severely impaired neurodevelopment (IQ < 50).[20,21] Unfortunately, the response to this incident did not include measuring internal iAs doses in affected children or establishing follow-up. Therefore, groundwork was not laid for more rigorous studies on the effects of acute iAs exposure during infancy.[20]

More recent studies have been conducted to establish the neurodevelopmental effects of iAs at much lower doses than those experienced by infants during the Morinaga, Japan dried milk poisoning. Numerous studies have demonstrated an inverse relationship between neurodevelopmental measures and iAs exposure.[22–24] For instance, using modified IQ tests in the Maternal and Infant Nutrition Intervention in Matlab (MINIMat) cohort in Bangladesh, researchers estimated that maternal and/or early childhood urinary iAs measures of 100 ppb are associated with a loss of 1–3 IQ points in Verbal and Full Scale IQ measures at 5 years of age. Interestingly, this

association was only significant for girls, but was seen with respect to iAs exposures occurring during early gestation, late gestation, and childhood.[25] Previous studies emerging from this population have not observed significant neurodevelopmental changes at time-points prior to 5 years of age, suggesting that these effects may emerge later in development.[26] In Maine, a cross-sectional study demonstrated that drinking well water with ≥5 ppb iAs was associated with a loss of 4–6 IQ points in Full Scale IQ and in the index scores for Perceptual Reasoning, Working Memory, and Verbal Comprehension, even after adjustment for covariates.[27] However, unlike the Morinaga dried milk poisoning in Japan, the populations under study did not have exposure that was limited to a single developmental window. As a result, researchers have not been able to isolate the effects of iAs exposure to a precise developmental window in humans. Nevertheless, these studies provide consistent evidence that prenatal and early life iAs exposure is associated with impaired neurodevelopment.

iAs-induced immunomodulatory effects are believed to underlie many iAs-associated health outcomes.[28] Disrupted immune functioning has been noted in adult populations with chronic iAs exposure.[29,30] With respect to early life exposures, research from the New Hampshire Birth Cohort, a prospective pregnancy cohort of women on private well water systems, provides evidence for similar outcomes in infants. In pregnant women exposed to low levels of iAs (average drinking water iAs level of 5.2 ppb), maternal urinary iAs was positively associated with infections in infants. In particular, prenatal iAs measures were associated with lower respiratory tract infections requiring prescriptions, and non-significantly associated with respiratory symptoms, upper respiratory infections, diarrhea symptoms, and infections in the first four months of life requiring a doctor's visit.[31] Updated results from this cohort show that these relationships persist at one year of age.[32] Similar evidence has also been reported in multiple Bangladeshi cohorts.[13,33,34] Again, it was not possible to pinpoint exposures that occurred only during the prenatal period, although there is evidence that ingested forms of iAs did not contribute significantly to iAs exposure during infancy.[35] Taken together, these results suggest that prenatal iAs is associated with impaired immunodevelopment. This is supported by molecular evidence demonstrating that prenatal iAs exposure is associated with disruptions in the expression of inflammatory-related genes, secretion of immune-mediating molecules, and shifts in lymphocyte subpopulations.[36–40]

With respect to reproductive outcomes, there is accumulating evidence that *in utero* iAs exposure is associated with adverse pregnancy outcomes, including miscarriage, fetal growth restriction, and gestational diabetes.[41–44] It has been demonstrated in several populations that prenatal iAs exposure is also associated with birth outcome measurements, including birth weight, head circumference and birth length.[41,45–49] Specifically, in the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort of women living in an iAs endemic region of Mexico, every one-unit increase in the percent of maternal urinary monomethylated arsenicals (%MMAs) correlated to a decrease of 24.4 grams in birth weight.[46] Exposure to MMAs, specifically, has also been linked to low birth weight in other populations.[45,46] Changes in such birth outcomes are significant given they may be early indicators of childhood IQ.[50,51]

At the molecular level, prenatal iAs exposure has been associated with large-scale gene expression changes in a population of iAs exposed infants in Thailand. In particular, pathways associated with inflammation and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) cascade were disrupted in association with iAs exposure corresponding to drinking water levels above the WHO 10 ppb recommended exposure limit.[36] In Bangladesh and Mexico, increases in protein expression of pro-inflammatory markers, including cytokines, have also been observed.[52,53] These transcriptomic and proteomic shifts in infants suggest that epigenetic mechanisms may control the response to prenatal and early life iAs exposure. Supporting this, in Bangladesh, it has been shown that prenatal iAs exposure is associated with changes in CpG methylation in genes enriched for functions specific to iAs-associated health outcomes, including those related to diabetes, immune functioning, cardiovascular outcomes, and cancer.[54] Moreover, there is evidence from the BEAR pregnancy cohort that suggests a role for both CpG methylation and miRNAs in the response to *in utero* iAs exposure. First, it was observed that newborn cord blood CpG methylation was significantly associated with drinking water iAs. This change in CpG methylation directly corresponded to functional changes in gene expression for a subset of genes with differential methylation. Moreover, several of these differentially methylated genes were significantly associated with birth weight, providing molecular-level evidence for the relationship between iAs and birth weight.[55] Second, miRNA expression was increased in the cord blood of these infants in association with prenatal exposure. These miRNAs were predicted to target 20% of the dysregulated gene expression observed in this population.[37] While there is not complete overlap between the specific dysregulated genes and epigenetic markers across each of these populations, this research has highlighted that iAs exposure leads to epigenetic reprogramming and dysregulated gene expression patterns, which may influence health outcomes in these populations and inform future mechanistic studies.

Using human studies to discern the precise developmental windows during which exposure yields specific iAs-associated diseases has obvious limitations. Even among studies where researchers can estimate prenatal or childhood exposures, it is often difficult to take into account exposures occurring during other developmental windows. This poses complications for the field, especially when considering the long latency of many iAs-associated health outcomes. As a result, there are wide gaps in our understanding of both the doses and developmental windows of exposure that yield such outcomes. Moreover, the ability to investigate the mechanisms underlying the wide array of iAs-associated diseases is limited in human studies. Such limitations underscore the need for animal models in

the study of iAs-induced disease. This is particularly important given the large populations of pregnant women and children at risk for elevated exposure across the world.

Health effects of prenatal and early life exposure: *in vivo* evidence

Due to metabolic differences between rodents and humans, there have been difficulties in establishing an animal model to investigate the various carcinogenic effects of iAs exposure.[56] However, mouse models using a prenatal and a "whole-life" exposure paradigm have been successfully established, allowing *in vivo* mechanistic studies of iAs-induced cancers.[57,58] The successes associated with using mouse models that include *in utero* exposure, but not those without, demonstrates the sensitivity of the prenatal period.

The prenatal exposure paradigm has been used to establish that prenatal iAs exposure is associated with several different types of cancers in adulthood. Following an exposure to 42.5 or 85 ppm $NaAsO_2$, from gestational day 8–18, offspring developed cancer at an increased incidence and multiplicity compared to controls. Interestingly, sex-specific effects were noted, with hepatocellular carcinomas and adrenal tumors in males, and cancers of the reproductive tract in females.[59] Offspring were also topically exposed to the tumor promotor 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which promoted iAs-induced liver tumors in females and lung tumors in both males and females.[59] Other research using this exposure paradigm demonstrated that prenatal exposure to 12.5 or 25 ppm of the trivalent monomethylated arsenical, methylarsonous acid ($MMAs^{III}$), induces many of the same lesions noted in iAs-exposed mice above, in a dose-dependent manner.[60] However, transplacental exposure to $MMAs^{III}$ also induced rarer testicular lesions, including interstitial and rete testis adenomas and hyperplasias.[60] These results may indicate that $MMAs^{III}$ is uniquely toxic, inducing different effects than iAs or other methylated iAs metabolites. This evidence mirrors observations in human populations where higher measures of urinary MMAs are associated with increased risks of cancers, skin abnormalities, chromosomal aberrations, and peripheral vascular disease, as reviewed by Tseng.[61] Additionally, as mentioned previously, %MMAs has been associated with a reduction in birth weight in infants with prenatal iAs exposure.[46] Together, these results suggest that MMAs may be more toxic compared to iAs and other iAs metabolites.

The prenatal exposure paradigm has also been used to provide mechanistic evidence for iAs-associated health effects. In particular, prenatal exposure to 85 ppm of $NaAsO_2$ has been noted to induce changes in methylation status of GC rich regions of hepatocellular DNA in C3H mice, along with an induction of glutathione system genes and metallothionein-1, a reduction in the expression of genes involved in methyl metabolism and the tumor suppressor gene p16, and disruption in CYP450s and insulin-like growth factor genes.[62] There is additional evidence that prenatal iAs exposure in male mice yields a 90% decrease in methylation of the estrogen receptor-α (ER-α) promoter, corresponding to a 3.1-fold increase in the expression of ER-α in iAs-induced hepatocellular carcinoma.[63] These findings provide evidence for the theory that epigenomic alterations may underlie the carcinogenic effects of iAs, providing a sustained reprogramming of cellular behavior necessary for the latency of many iAs-associated cancers.

Notably, the exposures required to study iAs-induced cancers in a prenatal mouse model are well above most upper limits of human exposure.[57–60,62,64,65] One of the reasons for the use of high doses is the observation that rodents are relatively resistant to iAs exposure compared to human populations.[65] Moreover, while a prenatal model is necessary to accurately determine which health outcomes are specifically associated with the prenatal exposure window, it often does not accurately reflect human exposures. Using a "whole-life" exposure paradigm, where CD1 mice were exposed to 50, 500, or 5000 ppb throughout gestation, adolescence, and adulthood, it has been demonstrated that exposures primarily in the 50 and 500 ppb range, but not 5000 ppb, are associated with lung cancers in both male and female mice.[64] In males, exposures in this range were associated with an increase in bronchiolo-alveolar tumors, while an increase in lung adenomas was observed in female mice.[64] These results correspond to evidence that suggests that iAs may serve as a chemotherapeutic at higher doses, but can be tumorigenic at lower doses.[64,66] The "whole-life" exposure paradigm has also been used to demonstrate that there is an overabundance of cancer stem cells (CSCs) in hepatocellular carcinomas and lung adenocarcinomas in CD1 mice exposed to 6, 12, or 24 ppm $NaAsO_2$, suggesting that iAs leads to the formation of CSCs in iAs-induced cancers.[58] Again, such findings present a possible explanation for the long latency associated with iAs-induced cancers by providing evidence for a population of cells that can remain quiescent following initiation.[67] Large populations of CSCs have also been noted in other iAs-exposed animal models and iAs-transformed cell lines, providing further evidence of the role of CSCs in iAs-induced cancers.[68–70] Moreover, many of the cancers identified using the "whole-life" exposure paradigm match, or are similar, to those identified in the prenatal mouse models, indicating that transplacental exposure may determine the target sites for carcinogensis.[58]

Aside from the carcinogenic effects of iAs exposure, prenatal and "whole-life" exposure paradigms have been employed to study other iAs-associated health effects, including cardiovascular, pulmonary, neurodevelopmental, metabolic, and reproductive effects, among others.[71–75] For instance, a recent study using a transplacental exposure paradigm found that exposure during the second half of gestation to iAs levels as low as 10 ppb was associated with increased body weight, impaired glucose tolerance and earlier onset of vaginal opening, an indicator of puberty, compared to controls.[74] Notably, effects were seen at much lower levels than those

required to investigate cancer outcomes in mice. However, the range of iAs-associated health outcomes that can be investigated using these exposure paradigms remains unclear. While mouse models allow researchers to isolate the precise windows of susceptibility associated with specific health outcomes, they may not offer a complete picture with respect to determining the doses at which these outcomes are observed in human populations, particularly for cancer outcomes. This is the result of metabolic differences between humans and mouse models, rendering humans more sensitive to iAs exposure. Nevertheless, these models provide valuable mechanistic data that is unobtainable from current human studies and demonstrates the ongoing need for animal research when studying the effects of prenatal and early life exposure to iAs.

Discussion and conclusions

iAs contaminated drinking water represents a global problem, putting developing fetuses and young children at risk of experiencing adverse health outcomes as the result of exposure. As the "natural experiment" in Antofagasta, Chile and the Morniaga, Japan dried milk poisoning incidents demonstrate, iAs exposure *in utero* and during early childhood is associated with unique health outcomes compared to exposures occurring during adulthood. These populations demonstrate the sensitivity of the prenatal and early life developmental periods and indicate that developing fetuses and young children are particularly at risk for disease associated with iAs exposure. However, these scenarios also represent some of the few situations in which it is possible to definitively isolate iAs exposure to specific developmental periods. Therefore, the need for animal models remains. Mouse models, including the prenatal and "whole-life" models better enable researchers to separate the effects associated with prenatal exposure from those associated with early life exposures. By using these exposure paradigms, the transplacental and early life effects of iAs can be more clearly differentiated.

Additionally, sex-dependent effects have been noted for iAs exposure in both human populations and mouse models. These results demonstrate that the sex of the infant influences the outcomes experienced following iAs exposure. As mentioned above, results from the MINIMat cohort demonstrate that IQ in females is significantly inversely associated with iAs exposure, but not in males.[25] Research from Antofagasta, Chile also demonstrates that the risk of cancer and non-cancer outcomes tends to be greater for males than for females.[11] In mouse models, some tumors are noted to be increased in incidence and multiplicity in both genders. However, in several studies, sex-dependent tumor locations have also been noted and provide more evidence that iAs acts in a sex-dependent manner.[57–60,64] Again, the prenatal and "whole-life" exposure paradigms allow for closer examination of the mechanisms driving such effects.

Despite the advances made in understanding the prenatal and early life effects of iAs exposure using available epidemiologic data and mouse models, important gaps in understanding remain. In particular, the dose ranges required for specific iAs-associated health outcomes have yet to be determined. For instance, the epidemiologic evidence for the neurodevelopmental impacts of iAs provides a mixed picture for the doses yielding a decrease in IQ in children. In the MINIMat cohort, where drinking water iAs is well above 10 ppb, the effect size reported estimates that a urinary iAs level of 100 ppb corresponds to a loss of 1–3 Full Scale IQ and Verbal IQ points in exposed children.[25] However, in Maine, exposure to drinking water levels greater than only 5 ppb iAs is associated with a decrease of 5–6 Full Scale IQ, Verbal Comprehension, Working Memory, and Perceptual Reasoning IQ points in children.[27] The disparity in these results may be related to several factors, including (1) nutritional differences between these populations,[76] (2) potential differences in susceptibility between populations with respect to the particular effects of iAs,[77] and possibly (3) difficulties in quantifying and comparing neurodevelopmental indicators, such as IQ, between different populations. The differences between the magnitude of these effects and possible underlying factors represent the challenge presented by identifying the critical dose ranges for iAs-associated health outcomes.

Unfortunately, due to the differences in sensitivity to iAs between humans and mouse models, there are complexities in extrapolating dose–response relationships between mice and humans, particularly for the carcinogenic effects of iAs.[65] Currently, the exposures required to induce cancers in mouse models, particularly using the prenatal exposure paradigm, often exceed those identified in human populations due to their relative resistance to iAs.[64,65] Thus, the current approach may be insufficient for defining these critical dose ranges. As discussed by Waalkes *et al.*,[65] the assumption cannot be made that equivalent drinking water exposures translates to equivalent internal doses for humans and mice. Instead, researchers should compare the amount of iAs that accumulates within target tissues, rather than comparing the levels of exposure between humans and mouse models. Such an approach may allow mouse models to be used more efficiently to study iAs-associated health effects and to determine the critical dose ranges responsible for iAs-induced disease.

Additionally, there is emerging evidence that epigenetic reprogramming may underlie the outcomes associated with iAs exposure. Epidemiologic evidence has contributed to this by demonstrating that both CpG methylation and miRNA expression are associated with iAs exposure in human populations. Moreover, a few studies demonstrate that these epigenetic markers correspond to functional changes in gene expression and represent a possible mechanism for iAs to reprogram cellular activities in a sustained manner, providing an explanation for the long latency periods between prenatal and early life exposures and adult-onset diseases.[37,54,55] Given the promise of inquiry into epigenetic mechanisms underlying iAs-induced effects, this research should be further expanded using animal models.

Looking forward, if iAs-induced diseases are epigenetically driven, it is possible that interventions may successfully

prevent the sustained cellular reprogramming observed following prenatal and early life iAs exposure, thereby protecting against iAs-induced disease. To date, nutritional interventions on iAs exposure have primarily focused on ensuring that nutritional deficiencies do not impede iAs metabolism.[76,78,79] However, the reversibility of epigenetic programming implies that nutritional interventions may also have the potential to alter the epigenetic dysregulation induced by iAs exposure.[80] Therefore, understanding the mechanisms underlying iAs-induced disease and the precise windows of developmental susceptibility in which they are observed is of great public health importance.

Conflict of interest

The authors declare no conflict of interests.

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