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Effects of *in utero* arsenic exposure on child immunity and morbidity in rural Bangladesh

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ABSTRACT

Chronic exposure to arsenic, a potent carcinogen and toxicant, via drinking water is a worldwide public health problem. Because little is known about early-life effects of arsenic on immunity, we evaluated the impact of *in utero* exposure on infant immune parameters and morbidity in a pilot study. Pregnant women were enrolled at 6–10 weeks of gestation in Matlab, a rural area of Bangladesh, extensively affected by arsenic contamination of tubewell water. Women ($n = 140$) delivering at local clinics were included in the study. Anthropometry and morbidity data of the pregnant women and their children, as well as infant thymic size by sonography were collected. Maternal urine and breast milk were collected for immune marker and arsenic assessment. Maternal urinary arsenic during pregnancy showed significant negative correlation with interleukin-7 (IL-7) and lactoferrin (Ltf) in breast milk and child thymic index (TI). Urinary arsenic was also positively associated with fever and diarrhea during pregnancy and acute respiratory infections (ARI) in the infants. The effect of arsenic exposure on ARI was only evident in male children. The findings suggest that *in utero* arsenic exposure impaired child thymic development and enhanced morbidity, probably via immunosuppression. The effect seemed to be partially gender dependent. Arsenic exposure also affected breast milk content of trophic factors and maternal morbidity.

1. Introduction

The presence of inorganic arsenic, a potent carcinogen and toxicant, in drinking water and crops is one of the major global environmental health problems. It is well documented that long-term arsenic exposure causes cancer and general toxicity in multiple organ systems (IARC, 2004; WHO/IPCS, 2001). Several studies of human exposure to arsenic in drinking water have shown adverse pregnancy outcomes in terms of decreased birth weight, increased rate of fetal loss, preterm births and neonatal mortality (Ahmad et al., 2001; Hopenhayn et al., 2003; Huyck et al., 2007; Rahman et al., 2007). Studies in animals have also shown arsenic exposure causing reproductive effects as well as transplacental carcinogenicity (Waalkes et al., 2007; Xie et al., 2007).

Part of the toxic effects of arsenic may be mediated by its effect on the immune system (Selgrade, 2007), e.g. by manifesting a state of immunosuppression that favors opportunistic infections. Recent studies in children showed associations between increased urinary arsenic levels and reduced proliferative response to mitogens, percentage of CD4 T cells and IL-2 secretion levels, suggesting immunosuppression (Soto-Pena et al., 2006). Also studies in animals have shown immunosuppressive effects of arsenic and increased susceptibility to infections (Aranyi et al., 1985). Prolonged exposure to arsenate in drinking water increases apoptosis of thymocytes and splenocytes in mice (Stepnik et al., 2005). *In vitro* studies have shown that arsenite decreases IL-2 mRNA expression, T cell activation, lymphocyte proliferation, impairs phagocytic properties of macrophages and induces cell cycle arrest and oxidative stress-mediated apoptosis (Conde et al., 2007; Lau et al., 2004; Lemarie et al., 2006). Considering the major species differences in arsenic kinetics (Vahter, 2002) these results need to be confirmed in humans.

In particular, little is known about the critical windows for arsenic-induced effects on immune response in children. Our recent large population-based study of effects of adverse pregnancy out-

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comes in relation to arsenic exposure through drinking water in Bangladesh showed increased infant mortality, particularly in infectious diseases (Rahman et al., 2007), indicating early effects of arsenic on immune function. Because arsenic easily crosses the placenta (Concha et al., 1998a) but not the mammary gland to breast milk (Concha et al., 1998b; Fängstrom et al., 2008), it may be hypothesized that the main immunotoxic effects are induced *in utero*. The present study aimed to evaluate effects of intrauterine arsenic exposure on thymic size and thymic function in children up to 1 year of age and on trophic factors in breast milk. The study is carried out in Matlab in rural Bangladesh, which is one of the areas that have been extensively affected by arsenic contamination of tubewell water (Rahman et al., 2006b; Vahter et al., 2006). A combined food and multi-micronutrient supplementation trial was carried out in Matlab to study effects on reproductive outcomes and infant development (Saha et al., 2008; Tofail et al., 2008). Within this cohort of pregnant women, the current study of the effect of arsenic exposure during pregnancy on immune response outcome in children was nested.

2. Materials and methods

2.1. Study area

The study area, Matlab, is located 53 km southeast of the capital Dhaka, in Bangladesh. Since 1966, the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) has run a health and demographic surveillance system (HDSS) in the Matlab area that now covers a population of about 220,000. Community health research workers visit every household on a monthly basis to collect information on demographic events, including marriage, pregnancy, birth, death, in- and out-migration, and morbidity of children below 5 years of age and of women of childbearing age. Socioeconomic information, including education and household assets, is also recorded by periodic censuses. In a recent study in Matlab, 13,286 tubewells were screened for the presence of arsenic (Rahman et al., 2006b). In total, 70% of the tubewells were found to exceed the WHO drinking water guideline for arsenic of 10 μg/L and 50% exceeded the national standard of 50 μg/L (Jakariya et al., 2007).

2.2. Study design and subjects

A randomized, large population-based food and multi-micronutrient supplementation trial (acronym the MINIMat study) was carried out in Matlab to study effects on size at birth, gestational age at birth, fetal loss and infant mortality (Persson et al, unpublished data; Saha et al., 2008; Tofail et al., 2008). In this study, through the monthly home visit by community health workers, pregnant women were identified by history of missed menstrual period. Upon identification of pregnancy, usually around 6–10 weeks of gestation, women were advised to visit their respective health facility in the area for confirmation of pregnancy by ultrasound and antenatal care. If fulfilling certain inclusion criteria (Rahman et al., 2008), the pregnant women were thereafter invited to be enrolled into the MINIMat trial. The first measurements were taken at the first clinic visit for ultrasound examination of 6–13 weeks. To adjust for measurement weeks, *Z*-scores were calculated for various measurements using international reference values (Chitty et al., 1994) and used for analysis. For convenience the first enrollment time was grouped together and referred to as 6–10 gw. The current study on the effect of arsenic exposure during pregnancy on immune response outcome in children was nested in the larger micronutrient supplementation trial. A 140 pregnant women delivering at the local health complexes were enrolled. Only singleton and full-term babies were included. The study was approved by the ethical review committee of ICDDR,B.

2.3. Arsenic exposure

The concentration of arsenic metabolites in urine is a suitable marker of ongoing exposure to inorganic arsenic, the main form in drinking water (Vahter, 2002). As arsenic easily crosses the placenta (Concha et al., 1998a), the concentration in maternal urine during pregnancy can be used as a proxy of fetal exposure. Following identification of pregnancy by urine test (about gestational week gw 6–10), women were asked to donate a urine sample for assessment of arsenic exposure. Another urine sample was collected in late pregnancy around gw 30 of women who were recruited in the MINIMat study and remained in the study at that point in time.

2.4. Anthropometry, morbidity and socioeconomic status

Birth weight was measured within 72 h of delivery, using electronic scales (electronic SECA pediatric scales) with precision of 10 g. Infant's recumbent length was measured using a regularly validated locally manufactured wooden length board, precision 0.1 cm. Infants' weight and length measurements were compared with those of the WHO Child Growth Standards [http://www.who.int/childgrowth/standards/en/] and converted to age and sex standardized *Z*-scores [BMI-for-age standard deviation scores in infants (BAZ)]. Anthropometric measurements at follow up (weight, height and mid upper arm circumference (MUAC), the mean of two measurements) were taken by trained field research assistants (FRAs). Weight was measured with portable electronic scale to nearest 100 g. Height was measured to nearest 0.1 cm with a locally made wooden height stick. Morbidity information of pregnant women was collected during home visits at regular intervals (gw 8, 14, 18, 22, 26, 30, 34 and 38) by Female Field Research Assistants (FFRA) using a set of questionnaire. Morbidity information included history of diarrhea, respiratory infections, including asthma and tuberculosis, and urinary tract infection at the time of the interview and within the previous 2 weeks. Morbidity information was also collected in a similar fashion for children at regular intervals up to 12 months of age. A case of acute respiratory infection (ARI) in children was defined by simultaneous presence of three criteria: (1) cough, (2) fever or shaking chills, and (3) fast breathing, difficulty breathing or chest retractions. Diarrhea was defined as passage of >4 loose stools in 24 h. On enrollment of the mothers; information was collected during a home visit to determine the socioeconomic status (SES). This information included their family wealth (number of possessions—e.g., television, radio, domestic animals, chairs, tables, beds, bicycle, or rickshaw), deficits between income and expenditure, family structure and parental characteristics (parents' education and employment), and housing quality (floor, walls, or roof made of mud).

2.5. Thymic index

Thymic size in infants was assessed by sonography, by trained medical doctor and paramedics using real-time ultrasound on a portable machine (Toshiba Portable System Model SSA328 Justavision-200, Tokyo, Japan) equipped with a 3.5-MHz convex transducer together with a PVF-745V 5.0/7.0 MHz sonographic microprobe. A validated method was used to assess thymic size in which the transverse diameter of the thymus and the saggital area of its largest lobe are multiplied to give a volume-related thymic index (TI) (Hasselbalch et al., 1996). Thymic size was assessed in children at 2, 6 and 12 months of age.

2.6. Specimen collection

Urine was collected by the pregnant women at 6–10 weeks and 30 weeks of gestation in plastic cups and then transferred to 24-mL polyethylene bottles. The samples were kept cool until transferred to the Matlab hospital laboratory at the end of the day, at the latest, where they were stored at −70 °C. The urine samples were transported frozen to the Karolinska Institute in Stockholm for analysis of arsenic metabolites. Breast milk was collected from mothers at 2, 6 and 12 months after delivery; the clear supernatant from milk after centrifugation was stored in −80 °C.

2.7. Arsenic concentrations in urine

The sum of the metabolites of inorganic arsenic in urine (inorganic arsenic + methylarsenic acid + dimethylarsenic acid) was determined using hydride generation atomic absorption spectrophotometry (Vahter et al., 2006). The results were expressed as μg/L of urinary arsenic (U-As), adjusted for variation in urine dilution by specific gravity, measured by a hand refractometer (ATAGO, Japan) (Vahter et al., 2006). The detection limit of this method is 1.3 ± 0.27 μg/L. For quality control purpose, certified reference materials from National Institute of Standards Technology (NIST 2670 urine HL and LL) with certified concentrations 480 ± 10 μg/L and 60 μg/L (not certified) were included in each analytical run. The obtained results were 505 ± 10 μg/L ($N = 23$) and 61 ± 2 μg/L ($N = 16$), respectively.

2.8. IL-7 and lactoferrin in breast milk

Interleukin-7 (IL-7) levels in breast milk were measured using DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The lower limit of detection was 0.1 pg/mL. Concentrations of lactoferrin (Ltf) in breast milk were determined using enzyme linked immunosorbant assay (OxisResearch™, OXIS International, Inc., Portland, OR) according to the manufacturer's instructions. The lower limit of detection was 1.6 ng/mL.

3. Statistical analysis

Statistical analyses were done using the statistical software SPSS 12.0 (2000 Apache Software Foundation). Normality as well as homogeneity of variances was checked. Spearman's correlation was done to evaluate association between U-As (at gw 6–10 and gw 30) and immune variables or morbidity outcomes. Signifi-