Toxicology and Applied Pharmacology 275 (2014) 22–27

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Mthfr gene ablation enhances susceptibility to arsenic prenatal toxicity

Bogdan J. Wlodarczyk *[,1], Huiping Zhu [1], Richard H. Finnell [1,2]

Center for Environmental and Genetic Medicine, Institute of Biosciences and Technology, Texas A&M University System Health Science Center, 2121 West Holcombe Blvd., Houston, TX 77030, USA

ARTICLE INFO

Article history:
Received 16 October 2013
Revised 3 December 2013
Accepted 18 December 2013
Available online 30 December 2013

Keywords:
Sodium arsenate
5,10-Methylenetetrahydrofolate reductase (MTHFR)
Mthfr knockout mice
Embryotoxicity
Gene–environment interaction
Teratogenicity

ABSTRACT

Background: In utero exposure to arsenic is known to adversely affect reproductive outcomes. Evidence of arsenic teratogenicity varies widely and depends on individual genotypic differences in sensitivity to As. In this study, we investigated the potential interaction between 5,10-methylenetetrahydrofolate reductase (*Mthfr*) genotype and arsenic embryotoxicity using the *Mthfr* knockout mouse model.

Methods: Pregnant dams were treated with sodium arsenate, and reproductive outcomes including: implantation, resorption, congenital malformation and fetal birth weight were recorded at E18.5.

Results: When the dams in $Mthfr^{+/-} \times Mthfr^{+/-}$ matings were treated with 7.2 mg/kg As, the resorption rate increased to 43.4%, from a background frequency of 7.2%. The As treatment also induced external malformations (40.9%) and significantly lowered the average fetal birth weight among fetuses, without any obvious toxic effect on the dam. When comparing the pregnancy outcomes resulting from different mating scenarios ($Mthfr^{+/+} \times Mthfr^{+/-}$, $Mthfr^{+/-} \times Mthfr^{+/-}$ and $Mthfr^{-/-} \times {}^{Mthfr+/-}$) and arsenic exposure; the resorption rate showed a linear relationship with the number of null alleles (0, 1 or 2) in the *Mthfr* dams. Fetuses from nullizygous dams had the highest rate of external malformations (43%) and lowest average birth weight. When comparing the outcomes of reciprocal matings (nullizygote × wild-type versus wild-type × nullizygote) after As treatment, the null dams showed significantly higher rates of resorptions and malformations, along with lower fetal birth weights.

Conclusions: Maternal genotype contributes to the sensitivity of As embryotoxicity in the *Mthfr* mouse model. The fetal genotype, however, does not appear to affect the reproductive outcome after in utero As exposure.

Published by Elsevier Inc.

Introduction

Arsenic is a naturally occurring element that exits in both organic and inorganic forms in the environment. Inorganic arsenicals, arsenite (trivalent) and arsenate (pentavalent) are the most commonly encountered forms in the environment. Human exposure to arsenic is primarily achieved through an oral route or inhalation from both natural and anthropogenic sources. For example, the introduction of arsenic into drinking water can occur as a result of its natural geological presence in local bedrock and cause serious consequences to human health. Anthropogenic sources of arsenic include the use of pesticides, feed additives, wood preserving arsenicals, mining activities and manufacture of electronic products (Wlodarczyk et al., 2011).

Arsenic is listed as number one on the Substance Priority List (SPL) of the 275 most hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR), highlighting the significant potential threat to human health due to its toxicity and potential for human exposure (http://www.atsdr.cdc.gov/SPL/index.html). Chronic exposure to arsenic impacts human health through its neurotoxicity, nephrotoxicity, hepatotoxicity and carcinogenicity (Singh et al., 2011). It accounts for the increased risk of various disorders such as cardiovascular abnormalities and diabetes mellitus (Navas-Acien et al., 2008).

Although assessment of its teratogenic potential in humans remains incomplete, suffering from a lack of large-scale epidemiological investigations, arsenic is known to induce congenital malformations, primarily neural tube defects (NTDs) in laboratory animals (Carter et al., 2003; Gilani and Alibhai, 1990; Leonard and Lauwerys, 1980; Machado et al., 1999). Animal studies have demonstrated that arsenic crosses the placenta and preferentially accumulates in the neuroepithelium of developing hamster, mouse and monkey embryos (Hanlon and Ferm, 1977; Lindgren et al., 1984). Our recent study demonstrated that maternal oral treatment with sodium arsenate induced NTDs in an inbred mouse strain, Lm/Bc/Fnn, which does not exhibit spontaneous neural tube malformations, yet is sensitive to arsenic's teratogenicity (Hill et al., 2008).

As indicated by the strain-specific sensitivity to teratogens like arsenic in mouse, it is generally hypothesized that gene–environment

Abbreviations: As, arsenic; Het, heterozygote; wt, wild type; Null, nullizygote; *Mthfr*, 5,10-methylenetetrahydrofolate reductase; neo allele, *Mthfr* gene allele with inserted neo cassette (containing neomycin resistance gene).

* Corresponding author at: Dell Pediatric Research Institute, The University of Texas at Austin, 1400 Barbara Jordan Blvd., Austin, TX 78723, USA. Fax: +1 512 495 4805.
E-mail address: bwlodarczyk@austin.utexas.edu (B.J. Wlodarczyk).
[1] Present address: Dell Pediatric Research Institute, Department of Nutritional Sciences, The University of Texas at Austin, Austin, TX 78723, USA.
[2] Present address: Dell Pediatric Research Institute, Department of Chemistry and Biochemistry, The University of Texas at Austin, Austin, TX 78723, USA.

0041-008X/$ – see front matter. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.taap.2013.12.014

interactions play important roles in the development of complex birth defects such as NTDs (Wlodarczyk et al., 2011). About two decades ago, a thermolabile variant caused by a transition of a single nucleotide was discovered (Jacques et al., 1996; Kang et al., 1988) in the human gene encoding the 5,10-methylenetetrahydrofolate reductase (MTHFR). This variant, *MTHFR* C677T, causes a 50–70% reduction in enzyme activity and intermediate levels of hyperhomocysteinemia (Jacques et al., 1996). The thermolabile allele (T) is heterogeneously distributed among different populations worldwide, with the frequency ranging from 12.6% among African-Americans to 46.0% among Campania Italians (Wilcken et al., 2003). Since its discovery, this common polymorphism has been implicated as a genetic modifier of a spectrum of folate preventable congenital malformations in a large number of epidemiology studies (Botto and Yang, 2000; Lupo et al., 2010; Nie et al., 2011; Shaw et al., 1998a, 1998b; Yin et al., 2012). The enzyme MTHFR is an important part of one carbon metabolism, catalyzing the conversion of 5,10-methylenetetrohydrofolate to 5-methyltetrohydrofolate, which is the methyl donor for methylation of homocysteine to methionine and then S-adenosylmethionine (SAM). SAM eventually serves as the principal methyl donor in many cellular metabolic processes, including the methylation of arsenic. Furthermore methylation of DNA and certain proteins (e.g. posttranslational modification of histones) is an important part of epigenetic regulation of gene expression. Disruption of this process during organogenesis can lead to embryonic death or congenital malformations. Because methylation is an important process of inorganic arsenic detoxification, and reduced methylation capacity is believed to increase arsenic related diseases (Tseng, 2007), it led us to speculate that the polymorphic *MTHFR* gene may contribute to the sensitivity of arsenic-induced congenital malformations. Additionally we have recently shown that different MTHFR activities significantly modulate arsenic excretion in mice (Wlodarczyk et al., 2012). We made use of a previously created *Mthfr* knockout mouse (Chen et al., 2001) to examine the potential interaction between genetic susceptibility conveyed by *Mthfr* gene, and in utero exposure of inorganic arsenic. The *Mthfr* knockout mice show full (wild type, $Mthfr^{+/+}$), intermediate (heterozygotes, $Mthfr^{+/-}$) or no MTHFR enzymatic activity (nullizygotes, $Mthfr^{-/-}$), respectively, which represents a valuable model of the human MTHFR C677T polymorphism.

Methods and materials

Animal husbandry. The *Mthfr* knockout mice that originated from Dr. Rima Rozen's laboratory were backcrossed to C57BL6/J mouse strain for at least ten generations. These mice were housed in the Institute of Biosciences and Technology Vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were maintained in clear polycarbonate microisolator cages and were allowed free access to food and water (Harlan Teklad Rodent Diet #8606, Ralston Purina, St. Louis MO). The mice were maintained on a 12-hour light/dark cycle. Nulligravid females, 50–70 days of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning, and the onset of gestation was considered to be 10 PM of the previous night, the midpoint of the dark cycle. In order to estimate the sensitivity of *Mthfr* knockout mice to prenatal arsenic exposure and to determine the possible interaction of fetal or maternal *Mthfr* genotypes, several mating scenarios were carried out: $Mthfr^{+/+} \times Mthfr^{+/-}$; $Mthfr^{+/-} \times Mthfr^{+/-}$; $Mthfr^{-/-} \times \mathrm{Mthfr}^{+/-}$; $Mthfr^{+/+} \times Mthfr^{-/-}$ and $Mthfr^{-/-} \times Mthfr^{+/+}$. All groups of dams used in these studies consisted of at least twelve mice (detailed numbers are provided in the results section and under the figures).

All experiments were performed on gravid dams. These studies were approved by the IBT Institutional Animal Care and Use Committee.

Arsenic treatment. Sodium arsenate (CAS# 10048-95-0, ACS Reagent, ≥98%, Sigma-Aldrich Chemicals, St. Louis, MO) was dissolved in water for injection (Sterile Water for Injection, USP, Abbott Laboratories Chicago IL) and administered by intraperitoneal (*ip*) injection at a dose volume of 10 μl/g body weight. Treatments were administered on gestational day (E)7.5 and 8.5, immediately preceding, and at the onset of, organogenesis (Leonard and Lauwerys, 1980). In the pilot study, sodium arsenate was tested at three dose levels: 9.6, 8.4 and 7.2 mg of As per kg of body weight. Based on the outcome of this study, the lowest tested dose, that is, 7.2 mg of arsenic was selected for further experiments. The control group was injected with Sterile Water for Injection at a volume of 10 μl/g body weight.

Observations and measurements. Gross maternal body weights were measured on the day E0.5 (plug day) and E18.5, when the animals were euthanized by CO_2 asphyxiation. The uterus was dissected out from the dam and numbers of implants, resorptions, live and dead fetuses as well as the fetal weight were determined and recorded. All viable fetuses were examined for external malformations. A tail tissue sample from each fetus was collected for genotyping. Genomic DNA was extracted using the DirectPCR Lysis Reagent (Viagen Biotech Inc., Los Angeles CA). *Mthfr* genotype was determined by PCR using forward primer 5′-GAC TAC CTG GCT ATC CTC TCA TCC-3′ and reverse primers (for wild type allele 5′-GAA GCA GAG GGA AGG AGG CTT CAG-3′; for neo allele 5′-AGC CTG AAG AAC GAG ATC AGC AGC-3′) followed by a 2% agarose gel electrophoresis. The PCR products were 145 bp and 216 bp long for the wild type and the mutant allele respectively.

Statistical methods. Non-parametric statistical test i.e. analysis of contingency table with Fisher's exact test was applied to compare the number of implantations, resorptions, dead and malformed fetuses between groups. For mean maternal and fetal weight evaluation, we used either a one-way Analysis of Variance (ANOVA) with the Tukey–Kramer multiple comparison test, or the Kruskal–Wallis test with the Dunn post-test, in case the group didn't encompass the normal distribution test. If only two groups were compared, an unpaired *t*-test was applied. For fetal genotype distribution, deviation from Hardy–Weinberg equilibrium (HWE) was evaluated using a chi-square "goodness of fit" test. All statistical analyses were conducted using GraphPad InStat (version 3.10; GraphPad Software, San Diego, CA), and the results of all tests were considered to be statistically significant when the p value (or adjusted p value) was less than or equal to 0.05.

Results

Reproductive outcomes in Mthfr Knockout mice

In order to assess maternal genotype effects on the reproductive outcome, untreated $Mthfr^{+/-}$ (het n = 14), $Mthfr^{-/-}$ (null n = 13) and $Mthfr^{+/+}$ (wt n = 12) female mice were mated with $Mthfr^{+/-}$ (het) male mice. There was no statistically significant difference in the occurrence of: implantations and resorptions among the three mating groups (Fig. 1A), and none of the collected fetuses had any external malformation. The average weight of fetuses from the null dams was significantly lower than those from the het dams ($p < 0.05$), but not lower than the wt dams. When fetuses from all matings were pooled and the genotypes were compared to each other, the average weight of the null fetuses was significantly lower than het and wt fetal weights ($p < 0.05$) (Fig. 1B). Among the het × het mating, the percentages of wt, het and null fetuses were 24.4%, 51.3% and 24.4%, respectively; no significant deviation from Hardy–Weinberg equilibrium (HWE) was observed ($p > 0.05$) (Fig. 3A).