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

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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.

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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).

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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).

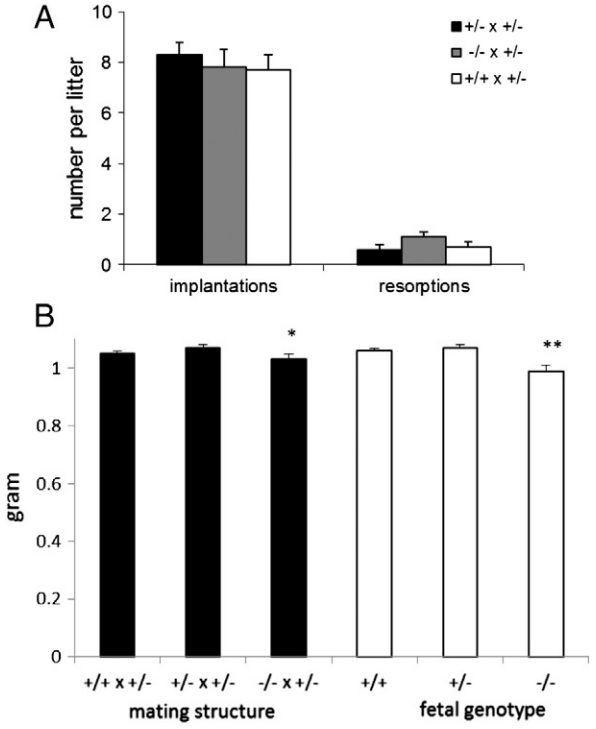

Fig. 1. A. Reproductive outcomes in untreated *Mthfr* mice with different genotypes. Number of dams per group: +/− × +/− (n = 14), −/− × +/− (n = 13), +/+ × +/− (n = 12). There were no significant statistical differences between groups. B. Mean weight of untreated E18.5 *Mthfr* fetuses. Black bars represent mean weight of all fetuses (different genotypes) from different mating scenarios. White bars represent mean weight of fetuses with specific genotypes from all mating scenarios. Number of fetuses: +/+ (n = 62), +/− (n = 150), −/− (n = 65). *Significantly different when compared +/− × +/− vs −/− × +/−. **Significantly different when compared −/− vs +/− and +/+.

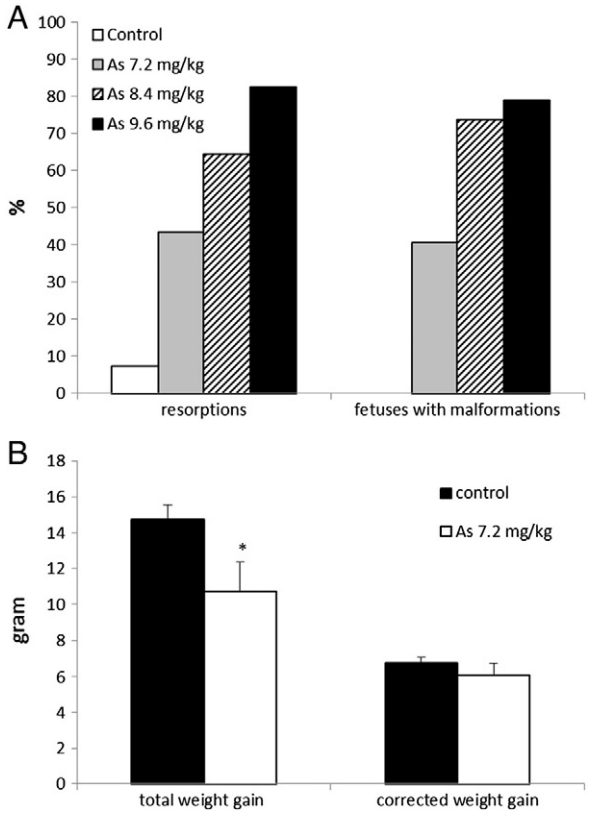

Fig. 2. A. Dose dependent embryotoxicity of sodium arsenate in *Mthfr* mice. Number of dams per group: As 9.6 mg/kg (n = 13), As 8.4 mg/kg (n = 12), As 7.2 mg/kg (n = 30), control (n = 14). A correlation coefficient test used to assess the linear relationship between the dose of arsenic and the reproductive outcome indicated that the observed dose–response was statistically significant ($p < 0.05$). B. Sodium arsenate effect on *Mthfr* dam's weight gain during pregnancy. Number of dams per group: As 7.2 mg/kg (n = 30) and control (n = 14). *Significantly different when compared to control group corrected weight gain = total weight gain − litter weight.

Dose-dependent embryotoxicity and teratogenic effect of Arsenic in Mthfr knockout mice

For assessment of As embryotoxicity, het × het matings were used. Four groups of pregnant dams were treated *ip* with 0 (water for injection) (n = 14), 7.2 mg/kg (n = 30), 8.4 mg/kg (n = 12) and 9.6 mg/kg (n = 13) of arsenic on E7.5 and E8.5. The resorption rate increased with the dose of As treatment, from 7.2% in controls to 82.6% at 9.6 mg of As/kg (linear regression, $p < 0.05$). Over 40% of the fetuses in the 7.2 mg/kg treatment group had external malformations; and the rates were even higher in the 8.4 mg/kg and 9.6 mg/kg groups (Fig. 2A). The results indicated the dose-dependent embryotoxicity of arsenic treatment, and demonstrated that the *Mthfr* knockout mice are considerably more sensitive to arsenic than other mouse strains (22, 23). For our subsequent experiments, the lowest dose of 7.2 mg/kg was used.

In this 7.2 mg/kg As dose group, the resorption rate was significantly increased compared to the control group (43.4% vs 7.2% respectively). In addition, 40.6% of fetuses (out of the 133 total live fetuses) exhibited external malformations. In these 54 affected fetuses we identified 87 major congenital defects including, from the most to least frequent: 23 exencephalies (26.4%), 23 craniofacial clefts (26.4%), 16 microphthalmia/anophthalmias (18.4%), 11 thoracoschisis/gastroschisis (12.6%), 7 brachygnathia (8.0%), 3 encephalocele (3.4%), 3 cleft lips (3.4%) and 1 cleft palate (1.1%). None of the fetuses from the 105 total control live fetuses exhibited any malformations. We subsequently compared the average weight between the fetuses with (n = 133) and without (n = 105) maternal As exposure. The average weight of the exposed fetuses was significantly lower than the unexposed controls ($p < 0.0001$). In order to determine whether As treatment also affected maternal weight, we compared the average weight gain of dams from gestation day 0.5 to 18.5. The As treated dams gained less weight than the un-treated control dams (one-tail *t*-test, $p < 0.05$). However, if the litter weight was deducted from the total weight gain (corrected weight), no difference was observed between treated and untreated dams (Fig. 2B).

Maternal Mthfr genotype is responsible for the sensitivity to As teratogenicity

In order to determine the possible effect of fetal and maternal *Mthfr* genotypes on As sensitivity, we conducted statistical analyses evaluating the relationships between the *Mthfr* null genotype and the prevalence of external malformations when exposed to As in utero. Initially, we examined the genotype distribution among all live fetuses from het × het matings with (n = 133) or without (n = 105) arsenic treatment (Fig. 3A). Among all live fetuses with arsenic exposure, the genotype distributions were 29.3%, 50.4% and 20.3% for wt, het and null fetuses, respectively. Although the frequency of null fetuses appeared to be lower than the non-treated group (24.4%), the reduction was not statistically significant (chi-square "goodness of fit" test, $p > 0.05$). No statistically significant difference was detected between the three different fetal genotypes in As treated group when comparing the number of malformed fetuses. Forty one percent of wt fetuses, 34% of het fetuses and 33% of null fetuses had external malformations (Fig. 3B).

When comparing reproductive outcome indices after As treatment among dams with different *Mthfr* genotypes (number of dams per group: 30 +/−, 13 −/− and 13 +/+) (males were +/− for all mating pairs), the resorption rates were significantly higher among het and

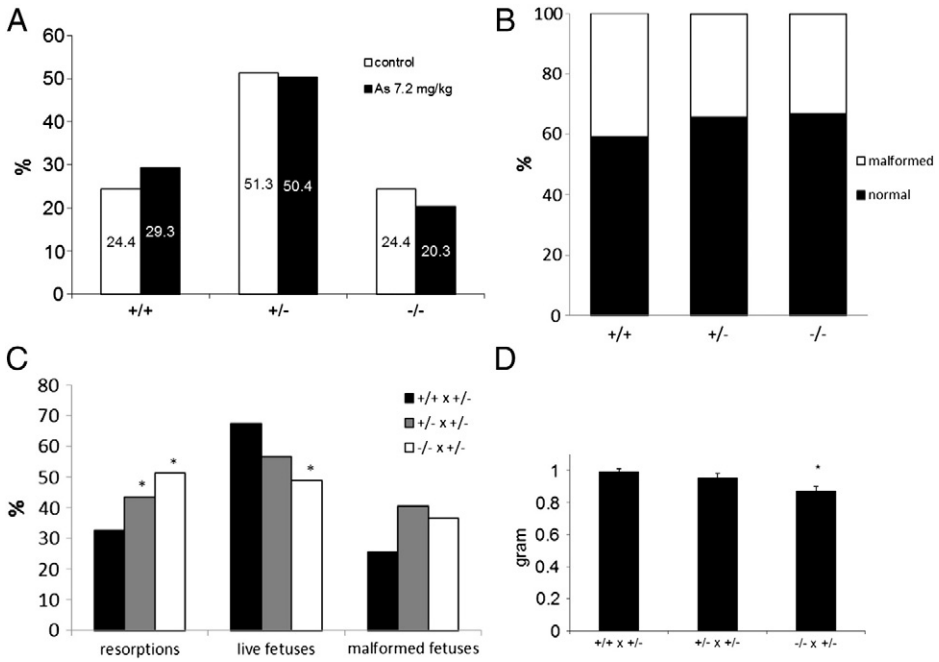

Fig. 3. A. *Mthfr* fetal genotype distribution from het × het mating. Number of dams per group: As 7.2 mg/kg (n = 30) and control (n = 14). No significant deviation from Hardy–Weinberg equilibrium (HWE) was observed ($p > 0.05$). B. Distribution of normal and malformed *Mthfr* fetuses based on their genotype from het × het mating. As 7.2 mg/kg *ip* on E 7.5 and 8.5. Number of dams per group: As 7.2 mg/kg (n = 30). C. Reproductive indices of dams with differing *Mthfr* genotypes treated with sodium arsenate. As 7.2 mg/kg *ip* on E 7.5 and 8.5. Number of dams per group: +/− × +/− (n = 30), −/− × +/− (n = 13), +/+ × +/− (n = 13). *Significantly different when compared to +/+ × +/− group. Resorption rate among the three groups showed significant linear correlation with the number of maternal null alleles. D. Average fetal weights from different mating structures following treatment with sodium arsenate. As 7.2 mg/kg *ip* on E 7.5 and 8.5.

null dams, and the average number of live fetuses was significantly less among null dams (Fig. 4C). Null dams also appeared to have fewer implantations, although the difference was not significant ($p > 0.05$). Because the different fetal genotype distributions among the progeny of the three mating types (wt × het, het × het and null × het) may contribute to the different reproductive outcomes of dams, we compared the resorption rates (number of resorption/numbers of implantation) among the three mating types, and in fact, the rate showed a significant linear correlation ($p < 0.05$) with the number of the null alleles (−) of the dam (Fig. 3C). We subsequently compared the frequencies of malformations among viable fetuses from the three mating groups. An increased malformation rate was observed in the het × het mating (40.6%) and in null × het mating (36.6%) compared to the wt × het mating (25.7%), although the difference was not statistically significant (Fisher exact test, $p > 0.05$). Comparison of the average fetal weight (including all fetal genotypes) among the three mating groups showed that fetuses born to null dams had significantly lower birth weights than those born to the wt dams treated with teratogenic concentrations of As ($p < 0.05$) (Fig. 3D).

In order to confirm the influence of the maternal genotype on As embryotoxicity, we set up reciprocal matings, wt × null and null × wt, where both mating types (15 dams in each) only produced heterozygous progeny. The dams were treated *ip* with As 7.2 mg/kg at E7.5 and E8.5. As shown in Figs. 4A and B, there were significantly fewer viable fetuses, lower fetal weights and more resorptions among the null × wt matings compared to wt × null matings ($p < 0.05$ for all comparisons). The nullizygous dams also had significantly fewer implantations per litter that the wild type dams (7.2 and 9.3, respectively). We further evaluated the potential synergistic effect of maternal and fetal *Mthfr* null genotype on As embryotoxicity. After As treatment, 9 null fetuses (36%) from As treated het dams had congenital malformations, while 7 null fetuses (46.7%) born to As treated null dams had malformations. In addition, the average birth weight of null fetuses born to null moms was significantly lower than null fetuses born to het dams (Fig. 4C). These results suggested that the maternal null genotype clearly plays an important role in As sensitivity in the *Mthfr* knockout mice. That is, our results demonstrated that maternal *Mthfr* genotype has significant effect on the reproductive outcomes measured by implantations number, resorption rate, malformation rate and birth weight after As treatment.

Discussion

To our knowledge, this is the first study modeling the gene–environment interaction between a commonly existing human genetic polymorphism (MTHFR C677T) and arsenic embryotoxicity using a well-suited mouse model. We demonstrated in our study that maternal *Mthfr* genotype alone does not significantly affect the reproductive outcome in untreated *Mthfr* mice. The only exception under normal conditions was the lower average weight of null live fetuses compared to het and wt fetuses; however, the *Mthfr* genotype did not affect the fetal viability.

We initially used a het × het mating strategy in order to assess As embryotoxicity in *Mthfr* mice. A relatively low dose of 7.2 mg/kg As (Wlodarczyk et al., 1996, 2001) reduced the fetal birth weight, induced response frequencies of 43% resorptions and 41% external malformations amongst viable fetuses, suggesting that these mice are highly susceptible to As embryotoxicity. These reproductive outcomes were observed at As concentrations that did not affect the maternal weight, suggesting that the treatment was not obviously toxic to the dams.

We subsequently analyzed the modulatory effects of fetal and maternal genotype separately in terms of in utero sensitivity to arsenic. Het × het matings produced fetuses of all three possible genotypes (null, het and wt). Arsenic treatment did not significantly affect the genotype distribution among the progeny. Null fetuses following As exposure had lower birth weights than did their het and wt littermates, but this was also true among fetuses without As exposure. In addition, fetuses of differing *Mthfr* genotypes showed similar response frequencies

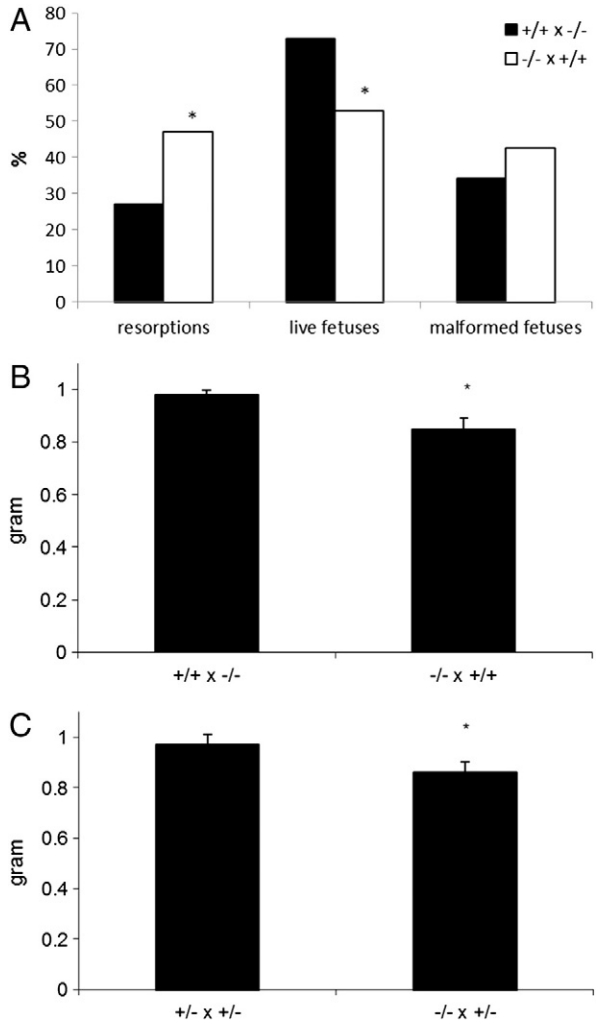

Fig. 4. A. Reproductive indices in *Mthfr* mice from reciprocal matings treated with sodium arsenate. As 7.2 mg/kg *ip* on E 7.5 and 8.5. Number of dams per group: +/+ × −/− (n = 15), −/− × +/+ (n = 15). *Significantly different when compared to +/+ × −/− group. B. Average fetal weight of *Mthfr* mice from reciprocal matings treated with sodium arsenate. As 7.2 mg/kg *ip* on E 7.5 and 8.5. Number of dams per group: +/+ × −/− (n = 15), −/− × +/+ (n = 15). *Significantly different when compared to +/+ × −/− group. C. Average weight of null fetuses from different mating structures treated with sodium arsenate. As 7.2 mg/kg *ip* on E 7.5 and 8.5. Number of dams per group: +/− × +/− (n = 30), −/− × +/− (n = 13). *Significantly different when compared to +/− × +/− group.

of arsenic induced external malformations. These results suggest that the fetal genotype contributes to As sensitivity minimally, if at all.

In order to evaluate the effect of maternal genotype on As sensitivity we compared reproductive outcomes secondary to As treatment among the three different mating types (female × male: wt × het, het × het and null × het). Since the male's genotype was consistent among all mating groups, the difference in As sensitivity mainly reflected the maternal genotype effect. The null dams had higher resorption rates, lower fetal birth weights, and higher prevalence of external malformations among viable fetuses, suggesting that the maternal *Mthfr* genotype contributes to the sensitivity of As-induced embryotoxicity. However, since the fetal genotype effect was not completely excluded, and all three genotypes could be found among the progeny of the het × het matings, we further confirmed the maternal effect by replicating the experiment in two reciprocal mating types, null × wt and wt × null, which both produced only heterozygous offspring.

The principal finding of our study is that the maternal MTHFR enzyme activity, as determined by the individual's genotype, contributes to the sensitivity of As induced embryotoxicity. It highlights the importance of genetic susceptibility in health problems caused by exposure to environmental toxins. The *MTHFR* C677T is considered a common human polymorphism which might be clinically relevant for a number of different human pathologies. For example, the frequency of the T allele, which is responsible for the reduced thermolability and activity of the MTHFR enzyme, is as high as 46% among some populations (Botto and Yang, 2000). The T allele effects multiple biochemical consequences altering the level of bioactive folate, homocysteine and other intermediate metabolites in the folate pathway, a shift in folate metabolism away from methyl group synthesis in favor of thymidylate synthesis (Quinlivan et al., 2005), and increased susceptibility to a variety of diseases and health conditions (Ueland et al., 2001). As a result, folic acid supplementation and fortification programs have been implemented in many countries, primarily for prevention of NTDs, although other health benefits have been observed. Given that the instability of the MTHFR enzyme among T allele carriers can be overcome by increasing their folate status, there is a legitimate concern that folic acid fortification may save homozygous TT fetuses that would otherwise be aborted, and subsequently lead to children being born with the TT genotype that favor certain pathologies throughout the lifespan (Smith et al., 2008). In some populations, an increase of T allele frequency due to "genetic selection" has already been linked to folic acid supplementation/fortification (Munoz-Moran et al., 1998; Reyes-Engel et al., 2002). The individuals with the TT genotype may, in fact, be more sensitive to environmental toxins such as arsenic. We have additionally observed that folate supplementation was able to rescue only a limited portion of As induced embryotoxicity in *Mthfr* mice (data not presented). This raises the concern that as more people become sensitive to environmental toxins like arsenic, folic acid simply won't be able to completely eliminate their genetically determined sensitivity. This speaks to the importance of gaining a further understanding of the underlying mechanisms in order to develop measures to prevent future As related diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by a NIH grant ES04917. We gratefully acknowledge the technical assistance of Hui Wei.

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