Methionine synthase reductase deficiency results in adverse reproductive outcomes and congenital heart defects in mice

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ARTICLE INFO

ABSTRACT

Low dietary folate and polymorphisms in genes of folate metabolism can influence risk for pregnancy complications and birth defects. Methionine synthase reductase (MTRR) is required for activation of methionine synthase, a folate- and vitamin B_{12}-dependent enzyme. A polymorphism in MTRR (p.I22M), present in the homozygous state in 25% of many populations, may increase risk for neural tube defects. To examine the impact of MTRR deficiency on early development and congenital heart defects, we used mice harboring a gene-trapped (gt) allele in Mtrr. Female mice ($Mtrr^{+/+}$, $Mtrr^{+/gt}$, and $Mtrr^{gt/gt}$) were mated with male $Mtrr^{+/g}$ mice. Reproductive outcomes and cardiac phenotype (presence of defects and myocardial thickness) were assessed at E14.5. Mtrr-deficient mothers had more resorptions and more delayed embryos per litter (resorptions per litter: 0.29 ± 0.13; 1.21 ± 0.41; 1.87 ± 0.38 and delayed embryos per litter: 0.07 ± 0.07; 0.14 ± 0.14; 0.60 ± 0.24 in $Mtrr^{+/+}$, $Mtrr^{+/gt}$, and $Mtrr^{gt/gt}$ mothers respectively). Placentae of $Mtrr^{gt/gt}$ mothers were smaller and their embryos were smaller, with myocardial hypoplasia and a higher incidence of ventricular septal defects (VSD) per litter (0; 0.57 ± 0.30; 1.57 ± 0.67 in $Mtrr^{+/+}$, $Mtrr^{+/gt}$, and $Mtrr^{gt/gt}$ groups respectively). Embryonic $Mtrr^{gt/gt}$ genotype was associated with reduced embryonic length, reduced embryonic and placental weight, and higher incidence of VSD, but did not affect myocardial thickness or embryonic delay. We conclude that Mtrr deficiency adversely impacts reproductive outcomes and cardiac development in mice. These findings may have implications for nutritional prevention of heart defects, particularly in women with the common MTRR polymorphism.

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Maternal periconceptional supplementation with pteroyl-monoglutamic acid (the synthetic form of folic acid) significantly decreases the risk of neural tube defects in offspring, and recent studies have suggested that it may also protect the embryo against the development of congenital heart defects (CHD) [1–4]. Risk for these defects may also be modified by polymorphisms in genes encoding enzymes in the folate metabolic pathway. The best-characterized polymorphism in folate metabolism is the c.677C → T mutation in methylenetetrahydrofolate reductase (MTHFR); several studies have suggested that this variant may increase the risk for CHD [5–8] MTHFR converts methylene-tetrahydrofolate (methyleneTHF) to methytetrahydrofolate (methylTHF), the primary form of folate in the circulation. Once transported into the cell, metabolized and converted into the polyglutamate form, methylTHF acts as the the carbon donor for homocysteine remethylation to yield methionine and tetrahydrofolate (THF). This reaction is catalyzed by methionine synthase encoded by the MTR gene. Methionine synthase is necessary for the buildup and maintenance of intracellular folate stores because it converts methylTHF (a poor substrate for polyglutamation which is prone to efflux from cells) to other folate forms that are good substrates for polyglutamylation and retention. For optimal and sustained activity, MTR is dependent on its cofactor (B_{12}) and on another enzyme, methionine synthase reductase (MTRR, EC 2.1.1.135), for activation. In earlier work, we identified a very common polymorphism in the methionine synthase reductase (MTRR) gene, c.66A → G (p.I22M), which is present in the homozygous state in approximately 25% of North Americans and Europeans [9]. This mutation, which affects the interaction between methionine synthase and methionine synthase reductase [10], has been examined in several clinical reports as a risk factor for NTD, with positive associations in some but not all studies [9,11,12]. However, few clinical studies have investigated the association between MTR/MTRR/vitamin B_{12} and risk for CHD. The only study to examine the effect of

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MTRR polymorphisms concluded that neither maternal nor case genotype significantly affected risk [13]. However, a study found that inadequate maternal intake of vitamin B$_{12}$, which leads to methionine synthase deficiency, was associated with increased risk for a CHD-affected child [14].

Congenital heart defects (CHD) affect approximately one in every 125 human births [15,16] and are the leading cause of birth defect-related deaths [17]. Successful morphogenesis of the heart in the developing embryo is dependent on the intricate coordination of many processes, including cellular differentiation, migration, proliferation, and apoptosis [18,19]. These processes may be impaired or modified by nutritional or genetic defects in folate metabolism due to the important role of folate in several cellular processes. 10-Formyltetrahydrofolate (formylTHF) and 5,10-methylenetetrahydrofolate (methyleneTHF) are necessary for the formation of purines and thymidylate, respectively. As mentioned above, methylTHF is utilized for the remethylation of homocysteine to form methionine. Methionine is required to generate *S*-adenosylmethionine (Ado-Met), the universal methyl donor for cellular methylation reactions. A ready supply of precursors for DNA replication and methionine for AdoMet production are known to be especially important for rapidly dividing cells, as demonstrated by the megaloblastic anemia caused by folate and B$_{12}$ deficiency, and the locus-specific anti-folate effects of methotrexate and 5-fluorouracil in cancer therapy. Disturbed balance between the folate forms and perturbation of cellular methylation capacity, whether caused by genetic and/or environmental factors, place the rapidly developing embryonic heart at greater risk for malformation [20]. Using our *Mthfr*-deficient mouse model, we demonstrated directly that disruption of the *Mthfr* gene or low dietary folate adversely affect the developing heart [1]. This finding is likely related to the fact that MTHFR deficiency in humans and mice is associated with low methylTHF, high homocysteine and reduced DNA methylation [21–23].

We recently reported the first characterization of a mouse model with *Mtrr* deficiency; this mouse has a gene-trap disruption in the *Mtrr* gene (*Mtrr^{Gt(pGT1Lxf)XG334Byg}*, hereafter abbreviated as *Mtrr^{gt}*) [24]. Compared with *Mtrr^{+/gt}* and *Mtrr^{+/+}* mice, *Mtrr^{gt/gt}* mice have elevated plasma homocysteine levels and lower plasma methionine levels. They also have non-significantly increased levels of methylTHF in both the liver and heart. We hypothesize that a mouse model with disruptions in the *Mtrr* gene would be useful for exploring the effects of disrupted folate and methionine metabolism on the development of CHD. In this report, we use this mouse model to directly examine the impact of *Mtrr* deficiency on the incidence of CHD as well as on other reproductive outcomes.

Materials and methods

Animals

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care. *Mtrr*-deficient mice were generated as reported [24] and back-crossed onto the C57Bl/6J background for 5–6 generations. Genotyping was performed as previously described [24]. Animals were fed commercial mouse chow diet 5001 (Agribrands Purina, St. Hubert, Canada) throughout mating and pregnancy. Three- to four-month-old *Mtrr^{+/+}*, *Mtrr^{+/gt}* and *Mtrr^{gt/gt}* female mice were housed overnight with *Mtrr^{+/gt}* males. Presence of a vaginal plug was designated gestational day (GD) 0.5. On GD 14.5, pregnant mice were euthanized by carbon dioxide asphyxiation and the uterus was removed and assessed for the number of implantation and resorption sites. Placentas were removed and weighed, and viable embryos were dissected and examined for gross external malformations, developmental delay, embryonic crown-rump length and weight as previously described [1]. Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4 °C and then transferred to 70% ethanol for storage. Yolk sacs were dissected and washed in PBS and used for embryonic *Mtrr* [24] and gender genotyping [25]. Fifteen litters per maternal genotype were obtained; 2 litters were removed from the study because of abnormally small litter size (1 embryo) leaving 14 litters in the *Mtrr^{+/+}* and *Mtrr^{+/gt}* groups.

Cardiac defects

As previously reported [1], embryos were dehydrated in an ethanol and xylene series and embedded in paraffin. Seven litters per maternal genotype were randomly selected and all the embryos of these litters were sectioned. Serial sections (6-μm-thick) were cut parallel to the longitudinal axis of the heart. All sections were examined under bright-field illumination with the use of an inverted microscope and assessed for the presence of cardiac defects.

Of the sectioned embryos, one embryo per mother was randomly selected for myocardial wall thickness measurement. Sections were stained with hematoxylin and eosin, photographed using an Axioplan Zeiss microscope and the compact region of the right and left ventricular myocardium was measured using AxioVision LE Image software. The thickness of compact walls was calculated as the mean of six measurements along the wall. All sections represented the same level along the longitudinal axis.

Statistical analysis

One-factor analysis of variance (followed by Tukey's post-hoc test) or independent sample *t*-tests were used to analyze parametric data (embryonic length, embryonic weight, placental weight, ventricular wall thickness). The Kruskal–Wallis test was used to analyze nonparametric data (number of resorptions per litter, delayed embryos per litter and embryos affected by VSD per litter). The χ^2 test was used to analyze embryonic genotype distributions. Fisher's exact test was used to assess differences in the number of delayed embryos and embryos affected by VSD. All statistical analyses were performed with SPSS for WINDOWS software (version 11.0; SPSS Inc., Chicago, IL). *P*-values <0.05 were considered significant.

Results

Reproductive outcomes

The results of reproductive outcomes grouped by maternal genotype are presented in Table 1. Maternal *Mtrr* genotype did not affect the number of implantation sites or the number of viable embryos per litter. However, the number of resorption sites per litter increased with disruption of *Mtrr* alleles, from 0.29 ± 0.13 in *Mtrr^{+/+}* mothers to 1.21 ± 0.41 in *Mtrr^{+/gt}* mothers to 1.87 ± 0.38 in *Mtrr^{gt/gt}* mothers (*P* = 0.002, Table 1). The genotype distributions of viable embryos (Tables 2 and 3) did not deviate significantly from expected Mendelian ratios in the different mating pairs, indicating that there was no preferential loss of any one genotype (Table 3).

Viable embryos were examined for gross morphology and overall delay by assessing the developmental characteristics that are present in normal E14.5 embryos. The outcome measures grouped by maternal genotype are shown in Table 1 whereas the same measures grouped by embryonic genotype are shown in Table 2. Table 3 presents the interaction between maternal and embryonic genotypes, by showing the outcomes for each embryonic genotype within the three maternal genotype groups. Maternal *Mtrr* genotype affected the number of delayed embryos per litter; a higher number of embryos of *Mtrr^{gt/gt}* mothers (0.60 ± 0.24) were delayed by a half-day to 1 day compared with the embryos of *Mtrr^{+/gt}* (0.14 ± 0.14) and *Mtrr^{+/+}* (0.07 ± 0.07) mothers (*P* < 0.05, Table 1). The incidence of delay was not influenced by either embryonic genotype alone (Table 2) or the interaction of embryonic genotype with maternal genotype (Table 3).

The size of the embryos, as assessed by length and weight, was influenced by both maternal and embryonic genotype. The embryos from *Mtrr^{gt/gt}* dams were significantly smaller than those from either *Mtrr^{+/gt}* or *Mtrr^{+/+}* dams (Table 1). In addition, *Mtrr^{gt/gt}* embryos were smaller than *Mtrr^{+/gt}* and *Mtrr^{+/+}* embryos, irrespective of maternal genotype (Table 2). Data in Table 3 suggest a genotype interaction for embryonic size. In *Mtrr^{+/gt}* dams, *Mtrr^{gt/gt}* embryos weighed less than either *Mtrr^{+/+}* or *Mtrr^{+/gt}* embryos and had a decreased length compared with *Mtrr^{+/gt}* embryos. In *Mtrr^{gt/gt}* dams, *Mtrr^{gt/gt}* embryos were smaller than *Mtrr^{+/gt}* littermates in terms of both length and weight. However, *Mtrr^{+/gt}* embryos of *Mtrr^{gt/gt}* dams were significantly smaller than *Mtrr^{+/gt}*