Low dietary choline and low dietary riboflavin during pregnancy influence reproductive outcomes and heart development in mice[1–3]

Jessica Chan, Liyuan Deng, Leonie G Mikael, Jian Yan, Laura Pickell, Qing Wu, Marie A Caudill, and Rima Rozen

ABSTRACT

Background: Embryonic development may be compromised by dietary and genetic disruptions in folate metabolism because of the critical role of folate in homocysteine metabolism, methylation, and nucleotide synthesis. Methylenetetrahydrofolate reductase (MTHFR), choline, and riboflavin play distinct roles in homocysteine detoxification and generation of one-carbon donors for methylation. The effect of low dietary choline and riboflavin on pregnancy complications and heart development has not been adequately addressed.

Objective: Our goal was to determine whether dietary deficiencies of choline and riboflavin in pregnant mice, with and without mild MTHFR deficiency, affect embryonic development.

Design: Female *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice were fed a control diet (CD), a choline-deficient diet (ChDD), or a riboflavin-deficient diet (RbDD) and were then mated with male *Mthfr*$^{+/-}$ mice. Embryos were collected 14.5 d postcoitum and examined for reproductive outcomes and cardiac defects.

Results: Plasma homocysteine was higher in ChDD- than in CD-fed females. Liver MTHFR enzyme activity was greater in ChDD-fed *Mthfr*$^{+/+}$ than in CD-fed *Mthfr*$^{+/+}$ females. The RbDD resulted in a higher percentage of delayed embryos and smaller embryos than did the CD. There were more heart defects, which were all ventricular septal defects, in embryos from the ChDD- and RbDD-fed females than from the CD-fed females. Dietary riboflavin and MTHFR deficiency resulted in decreased left ventricular wall thickness in embryonic hearts compared with embryos from CD-fed *Mthfr*$^{+/+}$ females.

Conclusions: Low dietary choline and riboflavin affect embryonic growth and cardiac development in mice. Adequate choline and riboflavin may also play a role in the prevention of these pregnancy complications in women. *Am J Clin Nutr* 2010;91:1035–43.

INTRODUCTION

Folates are involved in several critical one-carbon transfers that are important for development (1), including nucleotide synthesis and homocysteine remethylation to methionine for methylation reactions. Remethylation also serves to limit the concentrations of homocysteine, a potentially toxic amino acid. A key enzyme of folate metabolism—5,10-methylenetetrahydrofolate reductase (MTHFR)—converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF), which provides the one-carbon unit for folate-dependent remethylation of homocysteine (**Figure 1**). A common polymorphism of *MTHFR*, 677C→T (A222V), results in mild MTHFR deficiency and hyperhomocysteinemia in homozygous *TT* individuals (2). Hyperhomocysteinemia is also caused or exacerbated by low dietary folate intakes. Both hyperhomocysteinemia and mild MTHFR deficiency are associated with an increased risk of neural tube defects (NTDs) and cardiovascular disease (3–5). Some studies also suggest that low dietary folate and mild MTHFR deficiency may influence pregnancy complications and congenital heart defects, although additional studies are required (6–8).

We previously created a mouse model of MTHFR deficiency and showed that *Mthfr*$^{+/-}$ mice with a single null allele possess a biochemical phenotype that is similar to humans homozygous for the 677*TT* genotype (9). Using these mice, we investigated the effects of maternal mild MTHFR deficiency and low dietary folate on reproductive outcomes and heart development. We found that they contributed to increased reproductive loss and intrauterine growth delay or risk of congenital heart defects (10). However, the interaction between MTHFR and other micronutrients in the folate metabolic pathway, in the context of embryo and cardiac development, has not been studied.

Choline catabolism produces betaine, the methyl donor for folate-independent remethylation of homocysteine to methionine (Figure 1) (11). This reaction, catalyzed by betaine homocysteine methyltransferase (BHMT), becomes especially critical when MTHFR activity is compromised (11, 12). Choline is also converted to acetylcholine, and several studies have examined the effects of choline deficiency on neural tube development and brain function (13–17). One clinical study showed that higher choline intakes during pregnancy were correlated with a decreased risk of NTD in offspring, whereas studies in mice suggested that low choline intakes increased the incidence of NTDs (14, 16). However, no studies have examined the effects of choline deficiency on the developing heart.

[1] From the Departments of Human Genetics and Pediatrics, McGill University, Montreal Children's Hospital Research Institute, Montreal, Canada (JC, LD, LGM, LP, QW, and RR), and the Division of Nutritional Sciences, Cornell University, Ithaca, NY (JY and MAC).

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[3] Address requests for reprints and correspondence to R Rozen, Montreal Children's Hospital Research Institute, 4060 Ste Catherine West, Room 200, Montreal, Canada H3Z 2Z3. E-mail: rima.rozen@mcgill.ca.

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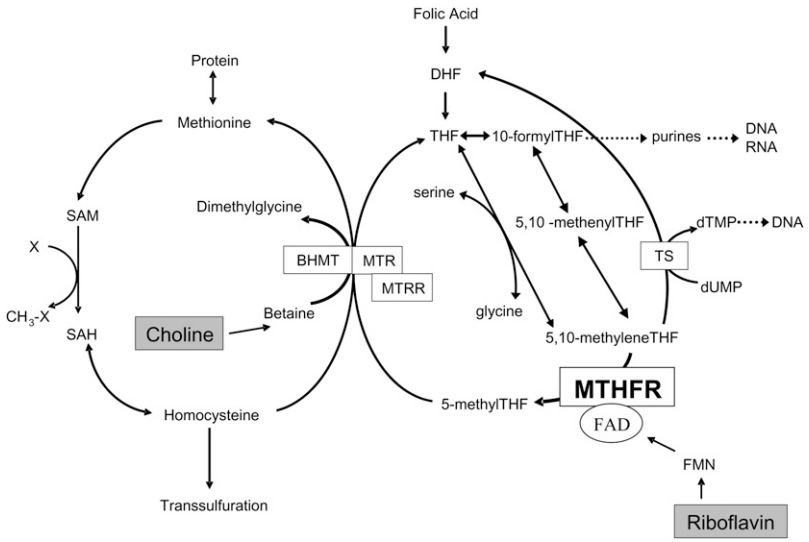

FIGURE 1. Folate metabolic pathway. 5,10-Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylene tetrahydrofolate (THF) to 5-methylTHF, a major methyl donor for the remethylation of homocysteine to methionine. Methionine synthase reductase (MTRR) is required for the recycling of methionine synthase (MTR), the enzyme that catalyzes the transfer of a methyl group from 5-methylTHF to homocysteine. Riboflavin is converted to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), the cofactor for MTHFR. Choline is the precursor for betaine, an alternate methyl donor in the remethylation of homocysteine to methionine by betaine-homocysteine methyltransferase (BHMT). DHF, dihydrofolate; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; TS, thymidylate synthase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate.

Riboflavin is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which function as cofactors for methionine synthase reductase (MTRR) and MTHFR, respectively (Figure 1). Clinical reports suggest that homozygous *TT* individuals with low riboflavin and low folate intakes have higher plasma homocysteine concentrations, which may be reduced by riboflavin supplementation (18, 19). Few clinical studies have examined riboflavin deficiency during pregnancy, although some rodent studies have shown that riboflavin depletion increases the incidence of congenital abnormalities, including ventricular septal defects (VSDs) (20, 21).

This study was conducted to determine the effect of low dietary choline and low dietary riboflavin during pregnancy, in the absence and the presence of mild MTHFR deficiency, on reproductive outcomes and congenital heart defects in mice.

MATERIALS AND METHODS

Mice and diets

Animal handling and experimentation were conducted according to the guidelines of the Canadian Council on Animal Care with approval by the Montreal Children's Hospital Animal Care Committee. MTHFR-deficient BALB/c mice were generated as reported, and genotyping was performed as previously described (9). On weaning at 4–5 wk of age, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females were fed amino acid–defined diets (Harlan Teklad, Indianapolis, IN) containing amounts of nutrients recommended by the American Institute of Nutrition (22). Mice were fed 1 of 3 diets: a control diet (CD) containing the recommended amount of folic acid, choline, and riboflavin (2, 2.5, and 6 mg/kg diet, respectively) or diets that were identical to the CD except for the

TABLE 1
Concentrations of maternal plasma choline/choline metabolites and riboflavin in pregnant *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ BALB/c mice fed control (CD), choline-deficient (ChDD), or riboflavin-deficient (RbDD) diets[1]

	CD diet		ChDD diet		RbDD diet		
	Mthfr$^{+/+}$	*Mthfr*$^{+/-}$	*Mthfr*$^{+/+}$	*Mthfr*$^{+/-}$	*Mthfr*$^{+/+}$	*Mthfr*$^{+/-}$	*P* value[2]
Choline (nmol/mL)	17.69 ± 1.2	20.63 ± 0.6	13.24 ± 1.3	13.16 ± 2.1	—	—	<0.01
Betaine (nmol/mL)	37.38 ± 3.7	40.31 ± 4.5	27.84 ± 3.8	25.09 ± 3.9	—	—	<0.01
Dimethylglycine (nmol/mL)	5.88 ± 0.7	6.71 ± 0.2	4.12 ± 0.6	4.16 ± 0.7	—	—	<0.01
Phosphatidylcholine (nmol/mL)	1627.71 ± 93.2	1647.17 ± 54.4	1085.93 ± 178.6	800.86 ± 152.4	—	—	<0.001
Lysophosphatidylcholine (nmol/mL)	291.1 ± 45	302.1 ± 7.1	208.98 ± 27.2	194.98 ± 34.5	—	—	<0.05
Glycerophosphocholine (nmol/mL)	47.74 ± 12.3	51.97 ± 9.6	27.1 ± 4.8	33.7 ± 6.4	—	—	<0.05
Phosphocholine (nmol/mL)	1.56 ± 0.16	1.79 ± 0.25	1.28 ± 0.17	1.44 ± 0.27	—	—	NS
Sphingomyelin (nmol/mL)	258 ± 9.5	244.25 ± 20.6	175.5 ± 33.8	217.5 ± 47.1	—	—	NS
Riboflavin (nmol/L)	19.65 ± 0.45	18.58 ± 1.06	—	—	1.9 ± 0.31	2.22 ± 0.26	<0.001

[1] All values are means ± SEMs; $n = 4$ per group. There were no significant effects of genotype or significant interactions between diet and genotype for choline/choline metabolites or riboflavin (ANOVA and *t* test).

[2] *P* values were derived from 2-factor ANOVA comparisons between the CD and ChDD groups and between the CD and RbDD groups.

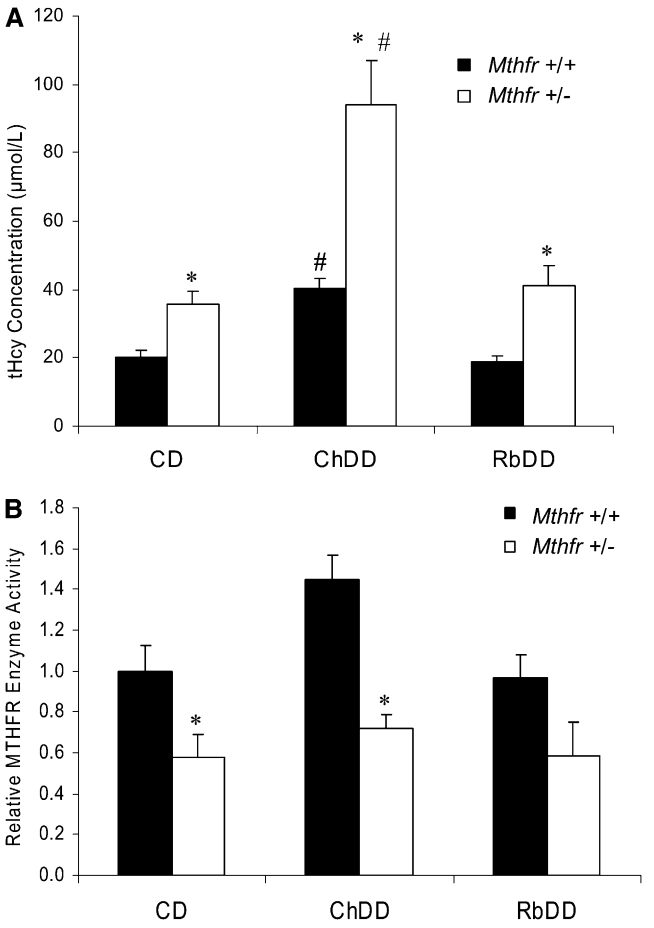

FIGURE 2. A: Mean (±SEM) plasma total homocysteine (tHcy) concentrations in pregnant mice fed a control diet (CD), a choline-deficient diet (ChDD), or a riboflavin-deficient diet (RbDD). $n = 6$ mice per group. $P < 0.001$ for genotype (ANOVA), $P < 0.001$ for choline deficiency compared with control diet (ANOVA), and $P < 0.05$ for significant interaction between genotype and choline deficiency (ANOVA). *Significantly different from *Mthfr*$^{+/+}$ females fed the same diet, $P < 0.01$ (t test). #Significantly different from CD-fed females of the same genotype, $P < 0.005$ (t test). B: Mean (±SEM) relative liver 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme activity in pregnant mice fed a CD, a ChDD, or an RbDD. n = 6–7 per group. All values are expressed relative to CD-fed *Mthfr*$^{+/+}$ females. $P < 0.05$ for ChDD compared with CD, and $P < 0.001$ for genotype (ANOVA). No significant diet-genotype interaction was observed. *Significantly different from *Mthfr*$^{+/+}$ females fed the same diet, $P < 0.001$ (t test).

content of choline and riboflavin [a choline-deficient diet (ChDD) containing one-eighth of the recommended amount of choline (0.3 g choline/kg diet) and a riboflavin-deficient diet (RbDD) containing one-sixth of the recommended amount of riboflavin (1 mg riboflavin/kg diet)]. The degree of choline and riboflavin deficiency was chosen based on previous studies (10, 23) in which a folic acid–deficient diet containing one-seventh of the recommended amount had resulted in hyperhomocysteinemia and increased adverse effects on reproduction and embryonic heart development.

All diets contained 1% succinyl sulfathiazole—an antibiotic that prevents de novo synthesis of folate by intestinal bacteria (24). After 6 wk of the diets, female mice were mated overnight with *Mthfr*$^{+/-}$ males aged 15–35 wk. Males were fed a standard nonpurified mouse diet (Agribands Purina, St-Hubert, Canada) and kept in isolated cages. The presence of a vaginal plug on the morning after mating was designated as 0.5 d post coitum (dpc). Females were fed their respective diets throughout breeding and pregnancy until 14.5 dpc, when they were injected with 5-bromo-2′-deoxyuridine (BrdU) 3.5 h before asphyxiation with carbon dioxide as previously reported (23). Blood was collected by cardiac puncture, and both uterine horns were removed for assessment of implantation and resorption sites. Nonviable or resorbed embryos at 14.5 dpc were considered as resorptions. The resorption rate was calculated as the number of resorption sites divided by the total number of implantation sites. Placentae were removed and weighed, and viable embryos were dissected and examined for gross abnormalities, developmental delay, and embryonic crown-rump length and weight as previously described (10). Developmental delay was assessed by comparison of the gross morphology of individual viable embryos with that of the most-developed embryo of each litter at 14.5 dpc. Embryos with incomplete separation of upper and lower digits, undefined elbows, a cone-shaped head, and absence of hair follicles were considered delayed. The percentage of delayed embryos was calculated as the number of delayed embryos per litter divided by the total number of viable embryos within that same litter. All embryos and placentae were fixed overnight in 4% paraformaldehyde and transferred to 70% ethanol for storage. Yolk sacs were removed and washed in phosphate-buffered saline before *Mthfr* genotyping as reported previously (10).

Measurements of plasma metabolites

Blood was collected into EDTA-coated tubes and centrifuged at 6000 × g for 6 min at 4°C. Plasma was separated from blood cells and frozen on dry ice before storage at –70°C. Liquid chromatography–mass spectrometry was used to measure plasma choline, betaine, and dimethylglycine (25); plasma phosphatidylcholine, lysophosphatidylcholine, glycerophosphocholine, phosphocholine, and sphingomyelin (26); and plasma riboflavin (27). Plasma total homocysteine (tHcy) was quantified with the A/C Portable Enzymatic Homocysteine Assay (A/C Diagnostics, San Diego, CA) and an A/C Diagnostics Reader (A/C Diagnostics) according to the manufacturer's instructions.

Liver protein extraction and MTHFR enzyme assays

Crude liver extracts were prepared, and MTHFR enzyme assays were performed as described previously (28, 29).

Histologic analysis of embryonic hearts

Approximately 10 litters from each of the 6 groups were randomly selected, and all embryos were processed overnight through a series of ethanol, xylene, and paraffin dehydration steps, each lasting 45 min. Embryos were then embedded in paraffin, and 6-μm serial transverse sections were collected. All sections were examined under bright-field illumination with the use of an inverted microscope. Sections were stained with hematoxylin (Sigma Aldrich, Oakville, Canada) and eosin (Sigma Aldrich) and photographed using an Axioplan Zeiss microscope.

One embryo per sectioned litter was randomly selected for measurement of myocardial wall thickness. Sections were stained with hematoxylin and eosin and measurements were taken by using AxioVision LE Image software. Because no atrial defects were observed, the focus was placed on measuring the thickness of the ventricular compact walls. This was calculated as the mean

TABLE 2
Effects of control (CD), choline-deficient (ChDD), and riboflavin-deficient (RbDD) diets on reproductive outcomes and ventricular septal defects (VSDs)[1]

	CD		ChDD		RbDD	
	Female *Mthfr*$^{+/+}$ genotype	Female *Mthfr*$^{+/-}$ genotype	Female *Mthfr*$^{+/+}$ genotype	Female *Mthfr*$^{+/-}$ genotype	Female *Mthfr*$^{+/+}$ genotype	Female *Mthfr*$^{+/-}$ genotype
Litters (*n*)	15	14	15	13	13	15
Viable embryos (*n* per litter)	6.00 ± 0.56[2]	4.93 ± 0.44	5.93 ± 0.42	5.38 ± 0.62	6.23 ± 0.60	6.33 ± 0.27
Resorption rate (*n* per litter [%])	27.15 (6.03)	24.17 (6.25)	26.56 (4.13)	26.06 (4.93)	30.12 (5.03)	16.47 (2.90)
Delayed embryos (%)	5.33 ± 3.50	8.65 ± 4.78	15.42 ± 6.32[3]	15.44 ± 6.01[3]	13.98 ± 4.11[4]	17.83 ± 3.81[4]
Mean embryonic weight (g)	0.205 ± 0.005	0.201 ± 0.005	0.196 ± 0.005	0.202 ± 0.003	0.193 ± 0.006[4]	0.192 ± 0.005[4]
Mean embryonic crown-rump length (mm)	10.684 ± 0.100	10.662 ± 0.080	10.530 ± 0.079	10.620 ± 0.116	10.464 ± 0.080[4]	10.469 ± 0.077[4]
Mean placenta weight (g)	0.104 ± 0.002	0.104 ± 0.002	0.107 ± 0.002	0.109 ± 0.002	0.100 ± 0.003	0.097 ± 0.006
Embryos with VSD (*n*/total sectioned [%])	3/60 (5.0)	3/54 (5.6)	9/54 (16.7)[5]	6/59 (10.2)[5]	12/56 (21.4)[5]	10/60 (16.7)[5]
VSDs (*n* per litter [%])	0.30 ± 0.15 (5.1)	0.27 ± 0.14 (5.1)	0.90 ± 0.31 (15.9)[4]	0.54 ± 0.25 (11.0)[4]	1.20 ± 0.36 (20.6)	1.00 ± 0.30 (17.6)

[1] There was no significant effect of maternal genotype when ChDD groups were compared with CD groups or when RbDD groups were compared with CD groups (2-factor ANOVA). No significant interaction was observed between diet and genotype for delayed embryos, embryonic weights, crown-rump lengths, or VSD incidence (ANOVA).
[2] Mean ± SEM (all such values).
[3] Fewer litters were examined for delayed embryos in the ChDD groups: 13 litters and 9 litters from ChDD *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females, respectively.
[4] Significantly different from CD females, $P < 0.05$ (2-factor ANOVA).
[5] Significantly different from CD females, $P < 0.05$ (Fisher's exact test).

of 3 values, measured independently by 2 individuals who were blinded to the groups; the correlation between individuals was 0.80 ($P < 0.01$). Sections were chosen to be along the same level of the longitudinal axis. BrdU staining was performed as previously described (23).

Statistical analysis

The female or litter was considered as the unit for statistical analysis. Results were expressed as means ± SEMs and examined by 2-factor analysis of variance (ANOVA) or independent-sample *t* tests. The chi-square test was used to analyze embryonic genotype distributions. The effects of embryonic genotype on embryonic length, weight, and placental weight were analyzed by using one-factor ANOVA. Tukey's test was performed if the ANOVA showed significance. The effects of embryonic genotype on CHD were analyzed by using the 2-tailed Fisher's exact test. All statistical analyses were performed with SPSS for WINDOWS software (version 11.0; SPSS Inc, Chicago, IL). *P* values <0.05 were considered significant, except for the analysis of interactions between diet and genotype, for which $P < 0.1$ was considered significant.

RESULTS

Plasma concentrations of choline/choline metabolites, riboflavin, and total homocysteine

Concentrations of plasma choline/choline metabolites and riboflavin are shown in **Table 1**. Mice fed the ChDD had significantly lower concentrations of plasma choline, betaine, dimethylglycine ($P < 0.01$, ANOVA), phosphatidylcholine

TABLE 3
Influence of embryonic *Mthfr* genotype on reproductive outcomes and the incidence of ventricular septal defects (VSDs) in female mice fed a control diet (CD)

	Female *Mthfr* genotype		Female *Mthfr* genotype		
	+/+		+/−		
Embryonic *Mthfr* genotype	+/+	+/−	+/+	+/−	−/−
Viable embryos (*n*)	37	53	17	34	18
Delayed embryos [*n* (%)][1]	3 (8.1)	5 (9.4)	4 (23.5)	2 (5.9)	0 (0)
Embryonic weight (g)	0.207 ± 0.005[2]	0.204 ± 0.003	0.195 ± 0.008	0.214 ± 0.004[3]	0.196 ± 0.005
Embryonic crown-rump length (mm)	10.673 ± 0.087	10.617 ± 0.068	10.571 ± 0.110	10.797 ± 0.086	10.667 ± 0.099
Placenta weight (g)	0.103 ± 0.002	0.102 ± 0.002	0.105 ± 0.004	0.100 ± 0.002	0.101 ± 0.002
Embryos with VSDs [*n*/total sectioned (%)]	2/23 (8.7)	1/37 (2.7)	2/16 (12.5)	1/24 (4.2)	0/14 (0)

[1] Significant difference from expected Mendelian ratios for number of delayed embryos from CD-fed *Mthfr*$^{+/-}$ female mice ($P < 0.05$, chi-square test).
[2] Mean ± SEM per litter (all such values).
[3] Significantly different from *Mthfr*$^{+/+}$ or *Mthfr*$^{-/-}$ embryos from CD-fed *Mthfr*$^{+/-}$ female mice, $P < 0.05$ (one-factor ANOVA followed by Tukey's test).

TABLE 4
Influence of embryonic *Mthfr* genotype on reproductive outcomes and incidence of ventricular septal defects (VSDs) in female mice fed a choline-deficient diet[1]

	Female *Mthfr* genotype		Female *Mthfr* genotype		
	+/+		+/−		
Embryonic *Mthfr* genotype	+/+	+/−	+/+	+/−	−/−
Viable embryos (*n*)	44	45	16	38	16
Delayed embryos [*n* (%)]	4 (9.1)[2]	5 (11.1)[2]	1 (6.2)[2]	3 (7.9)[2]	1 (6.2)[2]
Embryonic weight (g)	0.201 ± 0.003[3]	0.193 ± 0.007	0.207 ± 0.006	0.204 ± 0.004	0.196 ± 0.008
Embryonic crown-rump length (mm)	10.572 ± 0.072	10.338 ± 0.153	10.431 ± 0.153	10.695 ± 0.108	10.688 ± 0.149
Placenta weight (g)	0.106 ± 0.002	0.107 ± 0.004	0.112 ± 0.004	0.109 ± 0.003	0.104 ± 0.004
Embryos with VSDs [*n*/total sectioned (%)]	4/29 (13.8)	5/25 (20.0)	1/11 (9.1)	4/34 (11.8)	1/14 (7.1)

[1] No significant differences were observed by embryonic genotype (ANOVA and chi-square test).
[2] Fewer litters were examined for delayed embryos.
[3] Mean ± SEM per litter (all such values).

($P < 0.001$, ANOVA), and lysophosphatidylcholine and glycerophosphocholine ($P < 0.05$, ANOVA) than did mice fed the CD. No dietary differences were observed for phosphocholine and sphingomyelin, and there were no differences due to *Mthfr* genotype. Concentrations of plasma riboflavin were significantly lower in mice fed the RbDD than in mice fed the CD ($P < 0.001$, ANOVA), with no differences due to *Mthfr* genotype. These results indicate that the diets lowered the concentrations of the relevant nutrients as anticipated.

Plasma tHcy concentrations (**Figure 2**A) of pregnant CD $Mthfr^{+/+}$ and CD $Mthfr^{+/-}$ females were consistent with those reported previously (10). $Mthfr^{+/-}$ pregnant females had higher plasma tHcy concentrations than $Mthfr^{+/+}$ pregnant females overall ($P < 0.001$, ANOVA) and in each of the 3 dietary groups separately ($P < 0.01$, independent-samples *t* test). Females fed the ChDD had higher plasma tHcy concentrations than did the CD-fed mice ($P < 0.001$, ANOVA) and than their respective genotype groups fed the CD ($P < 0.005$, *t* tests). There was also a significant interaction between *Mthfr* genotype and choline deficiency ($P < 0.05$, ANOVA). ChDD $Mthfr^{+/-}$ females had the highest tHcy concentration of all groups (94.13 ± 12.83 μmol/L). There were no significant differences in tHcy concentrations between the RbDD-fed females and the CD-fed females.

Liver MTHFR enzyme activity

MTHFR enzyme activity was significantly decreased due to MTHFR deficiency ($P < 0.001$, 2-factor ANOVA; Figure 2B). Choline-deficient diets significantly increased enzyme activity ($P < 0.05$, 2-factor ANOVA), whereas there was no effect of riboflavin-deficient diets on activity (Figure 2B).

Reproductive outcomes

As shown in **Table 2**, there were no significant differences due to diet or genotype when resorption rates were compared between the CD and ChDD groups or between the CD and RbDD groups. No significant differences in the percentage of delayed embryos, embryonic weight, or length were observed between the CD and ChDD groups. On the other hand, RbDD females had a greater incidence of delayed embryos and lower embryonic weights and crown-rump lengths than did CD females ($P < 0.05$, 2-factor ANOVA).

Heart development

All the observed heart defects in embryos were VSDs (Table 2, **Tables 3–5**, and **Figure 3**). There was a greater number of embryos with VSDs in ChDD-fed females than in CD-fed females ($P < 0.05$, Fisher's exact test). The number of embryos with VSDs was significantly greater in the RbDD-fed groups than in the CD-fed groups ($P < 0.005$, Fisher's exact test), and the incidence of VSDs per litter was also significantly higher in embryos of RbDD-fed females due to diet than in those of CD-fed females ($P < 0.005$, 2-factor ANOVA). Maternal *Mthfr*

TABLE 5
Influence of embryonic *Mthfr* genotype on reproductive outcomes and incidence of ventricular septal defects (VSDs) in female mice fed a riboflavin-deficient diet[1]

	Female *Mthfr* genotype		Female *Mthfr* genotype		
	+/+		+/−		
Embryonic *Mthfr* genotype	+/+	+/−	+/+	+/−	−/−
Viable embryos (*n*)	47	34	24	47	24
Delayed embryos [*n* (%)]	9 (19.1)	6 (17.6)	2 (8.3)	11 (25.5)	7 (29.2)
Embryonic weight (g)	0.191 ± 0.005[2]	0.192 ± 0.006	0.197 ± 0.005	0.193 ± 0.005	0.193 ± 0.005
Embryonic crown-rump length (mm)	10.344 ± 0.105	10.513 ± 0.088	10.542 ± 0.101	10.521 ± 0.087	10.313 ± 0.121
Placenta weight (g)	0.098 ± 0.002	0.097 ± 0.003	0.099 ± 0.003	0.098 ± 0.002	0.097 ± 0.002
Embryos with VSDs [*n*/total sectioned (%)]	6/31 (19.3)	6/25 (24.0)	4/15 (26.7)	5/30 (16.7)	1/15 (6.7)

[1] No significant differences were observed by embryonic genotype (ANOVA and chi-square test).
[2] Mean ± SEM per litter (all such values).

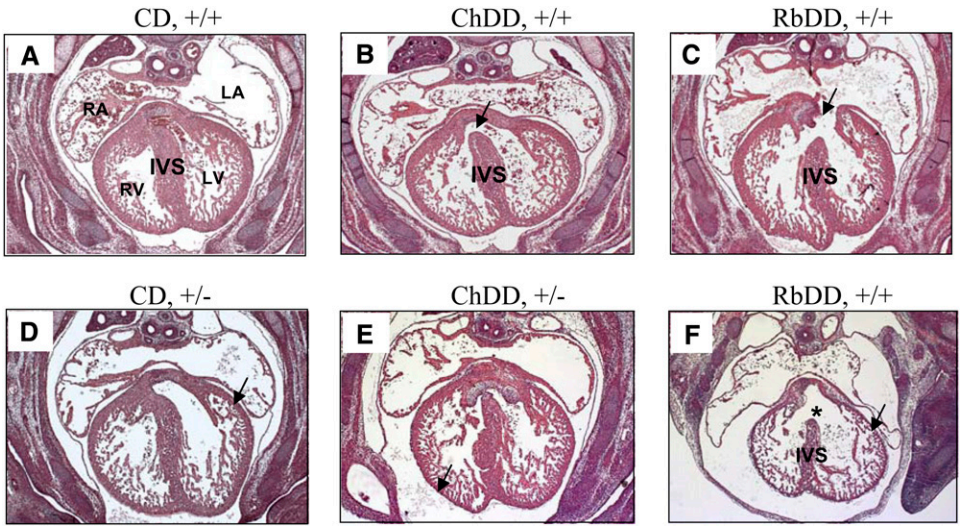

FIGURE 3. Influence of maternal 5,10-methylenetetrahydrofolate reductase deficiency, choline deficiency, and riboflavin deficiency on embryonic heart development in mice 14.5 d postcoitum (dpc). Representative transverse sections of the heart stained with hematoxylin and eosin are shown: normal embryonic heart at 14.5 dpc from *Mthfr*$^{+/+}$ female mice fed a control diet (CD; A), isolated ventricular septal defects (arrows) in *Mthfr*$^{+/+}$ female mice fed a choline-deficient diet (ChDD; B) or a riboflavin-deficient diet (RbDD; C), and embryonic hearts with thin ventricular myocardial walls in CD-fed *Mthfr*$^{+/-}$ mice (D) and in ChDD-fed *Mthfr*$^{+/-}$ mice (E) (arrows). Also shown is a malformed heart with a ventricular septal defect (asterisk) and thin ventricular walls (arrow) from an RbDD-fed *Mthfr*$^{+/+}$ female mouse (F). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; IVS, interventricular septum.

genotype did not influence the number of VSDs when *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females were compared within each diet group.

Thinner ventricular myocardial walls were observed in some embryos (Figure 3, D–F). For left ventricular wall thickness (**Figure 4**A), there was a significant interaction between diet and genotype when the ChDD-fed groups were compared with the CD-fed groups ($P = 0.08$) and when the RbDD-fed groups were compared with the CD-fed groups ($P = 0.07$). Maternal *Mthfr* deficiency was associated with thinner walls compared with *Mthfr*$^{+/+}$ females in the CD group ($P < 0.005$, *t* test), as indicated in Figure 4A. In the RbDD-fed group, maternal genotype resulted in lower left ventricular wall thickness (borderline significance: $P = 0.051$, 2-factor ANOVA). Left ventricular wall thickness was significantly lower in embryos from RbDD-fed *Mthfr*$^{+/+}$ females than in CD-fed *Mthfr*$^{+/+}$ females ($P < 0.05$, *t* test). There were no significant differences (2-factor ANOVA) in right ventricular wall thickness (Figure 4B). Cardiomyocyte proliferation was assessed through BrdU quantification, but there were no differences due to diet or genotype in any of the 6 groups (data not shown).

Effects of embryonic genotype

The distribution of embryonic *Mthfr* genotypes is shown in Tables 3–5 for female mice fed the CD, ChDD, and RbDD, respectively. No differences in numbers of viable embryos were seen between the observed and expected Mendelian ratios in any of the groups (chi-square test). In CD-fed *Mthfr*$^{+/-}$ females, *Mthfr*$^{+/-}$ embryos had higher weights than did either *Mthfr*$^{+/+}$ or *Mthfr*$^{-/-}$ embryos ($P < 0.05$, one-factor ANOVA). A significant difference was also observed in the Mendelian distribution of delayed embryos from CD-fed *Mthfr*$^{+/-}$ females, with a greater number of delayed *Mthfr*$^{+/+}$ embryos than *Mthfr*$^{+/-}$ embryos ($P < 0.05$, chi-square test); there were no delayed *Mthfr*$^{-/-}$ embryos. However, these observations may have been due to the small numbers of embryos examined within each genotype group. No other significant effects of embryonic genotype were observed on embryonic length, placental weight, or VSD incidence in the CD group. There were no effects of embryonic genotype for any variable in the ChDD-fed or RbDD-fed groups. These findings suggest that embryonic *Mthfr* genotype may not modify the risk of development of congenital heart defects; a similar conclusion was reached in our previous study (10). However, more litters would need to be examined to conclusively determine the effects of embryonic genotype on the abovementioned variables.

DISCUSSION

The current study presents novel data suggesting a relation between maternal choline deficiency and embryonic heart defects. We also showed that maternal riboflavin deficiency results in adverse reproductive outcomes and heart defects. Mild MTHFR deficiency and inadequate riboflavin intake may also affect heart development through an influence on ventricular wall thickness.

MTHFR deficiency alone did not appear to affect the incidence of resorption or heart defects, as compared with our previous findings (10). Nevertheless, in this study we observed that maternal MTHFR deficiency resulted in a significant decrease in left ventricular wall thickness in embryos from CD-fed females at 14.5 dpc. Although we had previously not observed a significant decrease in ventricular wall thickness in embryos from MTHFR-deficient females at 12.5 dpc, the additional 2 d of development in this study may have revealed an effect of MTHFR deficiency on wall thickness. The cause of the variability in the nature of the cardiac phenotype conferred by MTHFR deficiency remains to be determined.

The conversion of choline to betaine is important for the regulation of homocysteine concentrations, because betaine is an

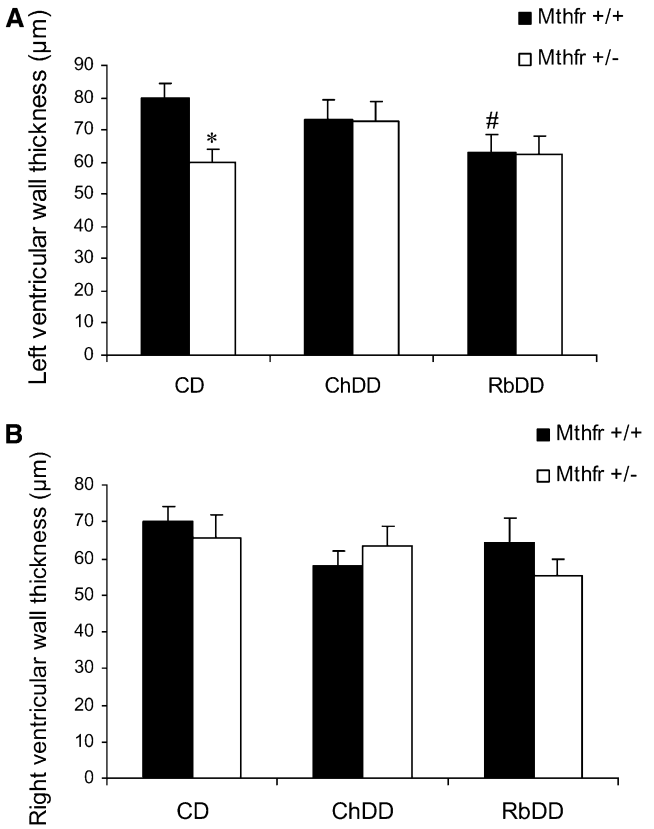

FIGURE 4. Mean (±SEM) influence of maternal 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency, choline deficiency, and riboflavin deficiency on embryonic left ventricular wall thickness (A) and right ventricular wall thickness (B) in mice 14.5 d post coitum. $n = 9–10$ per group. There was a significant interaction ($P = 0.08$) between diet and genotype between the mice fed a choline-deficient diet (ChDD) and those fed a control diet (CD) and between the mice fed a riboflavin-deficient diet (RbDD) and the mice fed a CD ($P = 0.07$). *Significantly different from CD-fed *Mthfr*$^{+/+}$ female mice, $P < 0.005$ (t test). Lower left ventricular wall thickness was nearly significantly different between the RbDD- and CD-fed female mice by genotype ($P = 0.051$, 2-factor ANOVA). #Significantly different from CD-fed *Mthfr*$^{+/+}$ females, $P < 0.05$ (t test). There were no significant differences or significant dietary-genotype interactions (ANOVA).

alternate methyl donor for homocysteine remethylation to methionine. This process becomes especially critical when folate-dependent remethylation is disrupted. We had previously shown that betaine supplementation decreased homocysteine concentrations in mice, regardless of MTHFR genotype, and that postnatal supplementation decreased mortality in *Mthfr*$^{-/-}$ offspring (11, 12). In humans, plasma homocysteine is negatively correlated with plasma betaine, because the flux through betaine-dependent homocysteine remethylation is increased when homocysteine is elevated (11). In the current study, low dietary choline resulted in higher homocysteine concentrations regardless of *MTHFR* genotype, with the greatest increase in *Mthfr*$^{+/-}$ females. The greater MTHFR enzyme activity in ChDD-fed *Mthfr*$^{+/+}$ females than in CD-fed mice may be a compensatory attempt to lower homocysteine concentrations by providing cells with a greater pool of 5-methylTHF. However, this did not return the homocysteine values to those seen in CD-fed *Mthfr*$^{+/+}$ females. Choline can also be converted to phosphatidylcholine in a reaction (phosphatidylethanolamine *N*-methyltransferase) that consumes *S*-adenosylmethionine and thus produces homocysteine (30). Consequently, there are several mechanisms by which choline can influence homocysteine concentrations. Interestingly, despite a large difference in plasma homocysteine concentrations, the incidence of VSDs in embryos from ChDD-fed *Mthfr*$^{+/-}$ females was not greater than that in ChDD-fed *Mthfr*$^{+/+}$ females, which suggests that elevated plasma homocysteine concentrations not be a critical factor or marker of abnormal heart development. However, maternal plasma homocysteine concentrations are not necessarily indicative of homocysteine concentrations in the developing embryo. The decrease in homocysteine remethylation due to choline deficiency would also lead to reduced levels of methyl donors for methylation reactions that are important for development and could result in decreased DNA methylation with altered gene expression. High homocysteine can also disturb methylation through its conversion to *S*-adenosylhomocysteine, a competitor of *S*-adenosylmethionine for methyltransferases (31, 32). It would be useful to study changes in gene expression and methylation in pregnant dams to assess whether these changes could influence the concentrations of critical metabolites or nutrients that are transferred to the fetus.

The greater incidence of VSDs in embryos from ChDD-fed females than in CD-fed females suggests that choline deficiency may affect cardiac structure. Of note, a number of embryos showed isolated VSDs without other abnormalities or features of delay ($n = 10/15$ isolated VSD), which suggests that the heart is especially sensitive to a ChDD. A study in the developing chick reported similar results, with homocysteine exposure causing isolated congenital heart defects and NTDs due to altered gene expression in neural crest cells (33). These findings support the hypothesis that high homocysteine concentrations affect the developing heart, but whether this is a direct effect of homocysteine or an effect on methylation and gene expression is unclear.

A clinical study found that changes in maternal choline metabolism may modify the risk of NTD in children (34). Other studies in cell and animal models have shown that choline deficiency caused changes in stem cell proliferation, global DNA methylation, and gene expression, which led to altered structure and development of neural tissues (16, 17, 35, 36). No studies thus far have been conducted on the effects of maternal choline deficiency on congenital heart defects. However, because both neural and cardiac tissues are composed in part of neural crest cells, it is not surprising that a reduction in choline intake would also affect heart development.

Clinical studies have shown that riboflavin intake can influence tHcy concentrations under certain conditions, including the concentrations in individuals with mild MTHFR deficiency due to the *677TT* genotype (18, 19). We showed that folate or FAD can stabilize both wild-type MTHFR or mutagenized MTHFR containing the polymorphism (37); consequently, the low-riboflavin diet could have influenced maternal tHcy or MTHFR activity. However, we did not observe an effect of RbDD on these factors. Because the RbDD contained both folic acid and choline, the effect of low riboflavin may have been limited.

MTHFR activity may not have been affected in this study because FAD can be more firmly bound by certain enzymes than others, even during riboflavin depletion (38). The incidence of delayed embryos was significantly increased in RbDD groups but these observations may or may not have been due to disturbances

related to homocysteine metabolism. Maternal plasma homocysteine was not affected by low riboflavin intake; however, as mentioned previously, maternal plasma homocysteine may not reflect embryonic concentrations. Changes in reproductive outcomes in riboflavin deficiency could have resulted from cellular malfunctions through a loss of FMN and FAD cofactors for other enzymes, such as the flavoproteins of the mitochondrial electron transport chain. It has been well-documented that mutations in mitochondrial DNA will lead to various disorders, including defects in cardiac and skeletal muscle (39–41). In this study, we found that embryos from RbDD-fed females also had a significantly increased incidence of VSDs, with many occurring in delayed embryos (n = 16/22 combined VSDs). If the RbDD-induced deficiency had indeed caused defective electron transport chain function, it would have resulted in the accumulation of reactive oxygen species and free radicals (42) and potential effects on apoptosis or cell growth. Thinner myocardial walls often lead to heart failure, and many studies point to mitochondrial defects as causes of a variety of cardiac conditions (43, 44).

Although no clinical reports have indicated riboflavin deficiency as a cause of congenital heart defects, several studies in rodents have suggested that riboflavin is important for heart development. Female rats fed an antagonist of riboflavin produced fewer offspring and many had VSDs, whereas fetal mice from riboflavin-deficient females had cardiovascular abnormalities and cleft palates (20, 21). An additional study conducted in galactoflavin-induced, riboflavin-deficient rats showed a marked decrease in liver FAD concentrations that was correlated with an increase in congenital malformations (45).

In conclusion, our findings emphasize the variable etiology of developmental disorders, which lends support to the importance of adequate dietary riboflavin and choline intake during pregnancy to decrease the risk of developmental defects and congenital heart defects.

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