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Infertility in 5,10-Methylenetetrahydrofolate Reductase (MTHFR)-Deficient Male Mice Is Partially Alleviated by Lifetime Dietary Betaine Supplementation[1]

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ABSTRACT

Metabolism of folate is essential for proper cellular function. Within the folate pathway, methylenetetrahydrofolate reductase (MTHFR) reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor for remethylation of homocysteine to methionine, the precursor of S-adenosylmethionine. S-adenosylmethionine is the methyl donor for numerous cellular reactions. In adult male mice, MTHFR levels are highest in the testis; this finding, in conjunction with recent clinical evidence, suggest an important role for MTHFR in spermatogenesis. Indeed, we show here that severe MTHFR deficiency in male mice results in abnormal spermatogenesis and infertility. Maternal oral administration of betaine, an alternative methyl donor, throughout pregnancy and nursing, resulted in improved testicular histology in *Mthfr*$^{-/-}$ offspring at Postnatal Day 6, but not at 8 mo of age. However, when betaine supplementation was maintained postweaning, testicular histology improved, and sperm numbers and fertility increased significantly. We postulate that the adverse effects of MTHFR deficiency on spermatogenesis, may, in part, be mediated by alterations in the transmethylation pathway and suggest that betaine supplementation may provide a means to bypass MTHFR deficiency and its adverse effects on spermatogenesis by maintaining normal methylation levels within male germ cells.

fertilization, gametogenesis, sperm, spermatogenesis, testis

INTRODUCTION

The folate cycle is essential for normal cell function and is involved in such processes as methionine production, and purine and pyrimidine synthesis. One crucial enzyme within the folate pathway is methylenetetrahydrofolate reductase (MTHFR). MTHFR irreversibly reduces 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF), the primary methyl donor for remethylation of homocysteine to methionine (Fig. 1). In turn, methionine provides the methyl group necessary for the formation of S-adenosylmethionine (SAM), which is involved in numerous cellular reactions including DNA, RNA, and histone methylation. One of the these methylation reactions, that of cytosine residues within DNA, is essential for development; the majority of mice deficient in any one of the DNA methyltransferase (DNMT) enzymes DNMT1, DNMT3a, or DNMT3b die before or soon after birth [1–3].

If MTHFR activity is eradicated in mice by gene-targeting, cellular levels of 5-methyl-THF, methionine, and SAM fall, and hypomethylation of DNA occurs [4]. Conversely, there are increased levels of homocysteine and S-adenosylhomocysteine (SAH), a potent inhibitor of DNA methylation [5]. Inhibition of MTHFR activity may also cause shunting of 5,10-methylene-THF toward the DNA synthesis pathway.

In humans, the common 677C→T polymorphism of *MTHFR* encodes a thermolabile enzyme with decreased activity that results in mild hyperhomocysteinemia when folate status is low [6–8]; the frequency of the homozygous mutant genotype is about 12% in the general Caucasian population [6, 7, 9, 10]. While individuals homozygous for this mutation are at increased risk of vascular disease, they appear to be protected against colon cancer and acute lymphocytic leukemia [11, 12]. Moreover, clinical evidence suggests that nearly 20% of men presenting at infertility clinics are homozygous for the *MTHFR* 677C→T polymorphism [13]; this rate was almost double the prevalence found in the control group. The latter findings suggest that mutations affecting MTHFR activity may underlie the pathology of some cases of male infertility. Furthering the hypothesis that MTHFR plays an active and important role in spermatogenesis is the observation that MTHFR activity is nearly five times higher in the adult testis than that in other major organs [4]. Indeed, as we show here, MTHFR deficiency results in abnormal spermatogenesis and male infertility.

One obstacle to studying male germ cell development in *Mthfr*-deficient mice is their limited survival to adulthood. *Mthfr*$^{-/-}$ mice have severely compromised survival, with less than 20% surviving beyond 3 wk of age [14]. However, dietary supplementation of betaine, provided to the mother throughout mating, pregnancy, and nursing, produces a substantial increase in *Mthfr*$^{-/-}$ pup survival, from 17% to 74%, and results in substantial improvement of various health and metabolite indices. Betaine, a choline derivative that has been used clinically to treat patients with MTHFR deficiency with some success [15, 16], is a substrate for betaine-homocysteine methyltransferase (BHMT), and serves as an alternate methyl donor for remethylation of homocysteine (Fig. 1) in the liver and kidney [17]. It is believed that deficiency in MTHFR may result in increased reliance on betaine-dependent remethylation; thus, provision of supplemental betaine may supply greatly needed additional methyl groups.

The purpose of the current study was to determine the role of MTHFR in spermatogenesis by examining the testes

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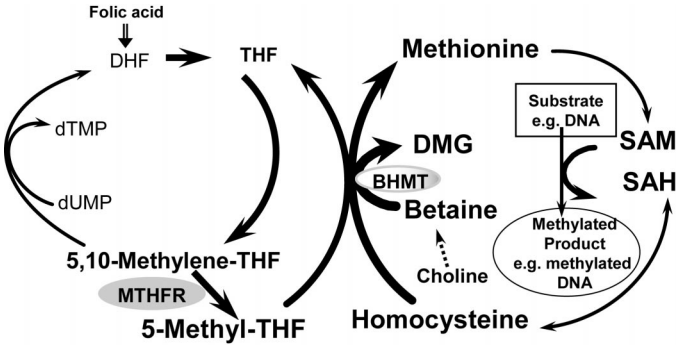

FIG. 1. The folate pathway, depicting the relationship between methylenetetrahydrofolate reductase, methionine, betaine, and DNA methylation. DHF, dihydrofolate; THF, tetrahydrofolate; DMG, dimethylglycine; BHMT, betaine-homocysteine methyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

of *Mthfr*$^{-/-}$ mice at different ages. Effects of short- and long-term betaine administration on sperm counts and fertility were also assessed. While MTHFR deficiency resulted in abnormalities in general development, severe oligospermia and infertility, long-term but not short-term betaine treatment, partially restored both spermatogenesis and fertility in *Mthfr*$^{-/-}$ male mice.

MATERIALS AND METHODS

Animals

Mice were maintained on a 12L:12D cycle and provided with food and water ad libitum. Mice were generated in our own breeding facility on a BALB/c background (Charles River Canada Inc, St. Constant, QC, Canada). Mice were from F6 to F8 generations, as a result of at least six generations of backcrosses to the BALB/c strain. All animal experimentation was conducted in accordance with the principles and procedures outlined by the Canadian Council for Animal Care. *Mthfr* genotypes were determined using a polymerase chain reaction-based assay [4].

MTHFR and Spermatogenesis—Experiment 1: Short-Term Betaine Treatment

In the first experiment the effects of MTHFR deficiency and short-term betaine supplementation on spermatogenesis were examined. *Mthfr*$^{+/-}$ males and females were mated to obtain male offspring of all genotypes (*Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$, *Mthfr*$^{-/-}$); 50% of mating females were assigned a diet of water and regular mouse chow (Purina Laboratory Rodent Diet 5001, Purina Mills) and the remaining females were provided with regular mouse chow and 2% betaine-supplemented water (Sigma) during the time of mating, pregnancy, and nursing (short-term betaine). At weaning, all pups were given water and regular mouse chow. The 2% oral betaine concentration resulted in an intake of approximately 3 g per kilogram body weight of the dams. This dosage was based on previous studies [14, 18] in which increasing the oral betaine beyond 2% improved phenotypes and metabolic indices only minimally.

Male offspring were killed at 6 days postpartum (dpp) (control: *Mthfr*$^{+/+}$, n = 4; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 4; short-term betaine: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 7; *Mthfr*$^{-/-}$, n = 4) and at 8 mo of age (control: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 9; *Mthfr*$^{-/-}$, n = 4; short-term betaine: *Mthfr*$^{+/+}$, n = 3; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 5), with the day of birth designated as 0 dpp. Blood was collected and the testes were removed and weighed. The left testis was snap-frozen and stored at −80°C, and the right testis was immersed in formalin or Bouin fixative (BDH Inc., Toronto, ON) for 5 to 24 h, dehydrated, and embedded in paraffin for histological analysis. Sections (5 μm) were cut, mounted on glass slides, deparaffinized with xylene, and stained with hematoxylin-eosin. Slides were viewed with a Zeiss Axiophot photomicroscope and pictures were taken with a SPOT RT Slider digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Additionally, the effects of MTHFR deficiency on early spermatogenesis were also examined at E18.5 (day of plug = Embryonic Day 0.5, E0.5). Pregnant females, all given supplemental betaine, were killed at 18.5 days of gestation and caesarian surgeries were performed. Testes of male embryos were collected in Bouin fixative, processed, cut into serial sections (5 μm), and used for germ cell quantification. DNA from embryo head samples was used to genotype embryos.

Detection of Germ Cells

The monoclonal germ-cell nuclear antigen antibody, GCNA1, specifically recognizes germ cells and was used to identify germ cells on Bouin- or formalin-fixed testes collected at E18.5 and 6 dpp [19]. Sections from E18.5 testes were cut into serial sections, with every fifth section used for germ cell quantification. For both E18.5 and 6 dpp testes, sections were incubated overnight with undiluted primary antibody at 4°C. The Elite ABC Kit protocol (Vector Laboratories, Burlington, ON) was used for blocking and primary antibody detection, with the exception that the biotinylated antibody and Vectastain Elite ABC reagent incubations were extended to 2 h on 6 dpp testes fixed in formalin. For E18.5 testes, germ cells were counted by an individual blinded to the slide identities and were reported per testis and per 2000 Sertoli cells as described by Nadler and Braun [20].

Detection of Apoptotic Germ Cells

Germ cell apoptosis at 6 dpp was examined using the TUNEL detection protocol (Apoptag Kit; Intergen Co., Purchase, NY) according to the manufacturer's directions, on formalin- or Bouin-fixed testes. The following modifications were used for formalin-fixed testes: terminal deoxynucleotidyl transferase enzyme incubation, 95 min; antiperoxidase incubation, 60 min; diaminobenzidine developing time, 15 min; and hematoxylin staining, 45 sec. TUNEL-positive cells were quantified and reported as the number of TUNEL-positive cells per 100 tubules.

Quantification of Abnormal Seminiferous Tubules

Slides were viewed by light microscopy and tubules were staged according to the method described by Oakberg [21]. Abnormal tubules were quantified and expressed as a percentage of total tubules examined (100 tubules counted per animal).

Betaine and Maintenance of Spermatogenesis—Experiment 2: Short-Term Versus Long-Term Betaine Treatment

Betaine has been shown to substantially improve the health and phenotype of *Mthfr*$^{-/-}$ mice [14, 18]. Thus, we hypothesized that betaine would have a similar positive effect on male germ cell development in *Mthfr*$^{-/-}$ males. To do so, a second experiment was conducted in which all mating females were provided with a 2% oral betaine solution during the time of mating, pregnancy, and nursing, and betaine treatment was then continued postweaning in half of the mice. Pups were weaned at 24 days and males of each genotype were randomly assigned to an amino acid-defined dry diet (control; TD 01463; Harlan Teklad, Madison, WI) with no betaine, or the same diet but containing 2.93 g/kg supplemental betaine (TD 01462; Harland Teklad), resulting in an oral betaine intake of approximately 300 to 350 mg/kg body weight (short-term betaine: *Mthfr*$^{+/+}$, n = 8; *Mthfr*$^{+/-}$, n = 11; *Mthfr*$^{-/-}$, n = 3; long-term betaine: *Mthfr*$^{+/+}$, n = 6; *Mthfr*$^{+/-}$, n = 7; *Mthfr*$^{-/-}$, n = 5). At 3 mo of age, all males were mated. Following mating, males were killed and testes, epididymides, seminal vesicles, spleen, and liver were removed, weighed, snap-frozen, and stored at −80°C. The right testis was placed in Bouin fixative and prepared for histological analysis.

Sperm Counts

Hemocytometric counts of testicular spermatozoa were performed according to the method described by Robb et al. [22], with modifications [23].

Fertility Analysis

The effect of MTHFR-deficiency and extended betaine supplementation on male fertility was examined by mating each male to two virgin CD1 females (Charles River Canada, Inc.) over the course of 5 days. Females were examined daily for vaginal plugs. The following week, mating was repeated with another two virgin CD1 females. Mating behavior, pregnancy rate (the number of plug-positive females that became pregnant), litter size, and sex ratio were determined. All fertility data were calculated and are presented on a per male basis.

Statistical Analyses

Data were examined statistically using two-way analysis of variance, followed by the Tukey test (Sigma Stat; SPSS, Chicago, IL). Nonparametric tests were applied when sample sizes were too small to formally test for normal distribution. A Fisher exact test was used to analyze fertility data. The level of significance for all analyses was $P \leq 0.05$.

RESULTS

General Effects

In addition to examining the role of MTHFR in spermatogenesis, the first experiment was designed to determine whether betaine, while improving pup survival and liver and brain phenotype [14], could remedy any MTHFR-deficiency-mediated effects on male germ cell development. As previously reported, there were no differences in litter size between supplemented and nonsupplemented females, and withdrawal of betaine at weaning did not affect *Mthfr*$^{-/-}$ pup survival [14]. In both experiments reported here, body weight was monitored as a general gauge of health. For body weight and other end points evaluated, *Mthfr*$^{+/+}$ or *Mthfr*$^{+/-}$ males did not differ, both within and between diets, unless specified.

As shown in Table 1, the average body weight of untreated 6 dpp *Mthfr*$^{-/-}$ males was only 37% ($P < 0.001$) of the weight of untreated *Mthfr*$^{+/+}$ males. The body weight of *Mthfr*$^{-/-}$ mice was also significantly lower than that of betaine-supplemented *Mthfr*$^{-/-}$ males, but otherwise, control-diet *Mthfr*$^{-/-}$ males appeared healthy. In adults, mean body weight of control *Mthfr*$^{-/-}$ males was not different from that of control-diet *Mthfr*$^{+/+}$ males ($P = 0.449$), yet *Mthfr*$^{-/-}$ males given short-term betaine weighed significantly less ($P < 0.05$) than their *Mthfr*$^{+/+}$ counterparts. White blood cell counts and hemoglobin levels did not differ across genotype or diet (data not shown).

MTHFR Deficiency and Betaine to Weaning on Male Reproductive System (Experiment 1: Short-Term Betaine)

Effects on male reproductive organ weights at 6 dpp and 8 mo. The effects of MTHFR deficiency on testis weight in neonates and adults are shown in Figure 2, A and B. Despite lowered body weights, the mean relative testis weights of *Mthfr*$^{-/-}$ males did not differ with either diet from their *Mthfr*$^{+/+}$ controls (Fig. 2A). In adult males, however, MTHFR-deficiency resulted in considerably reduced testis weights (Fig. 2B). The relative testis weights of *Mthfr*$^{-/-}$ mice of either diet were less than one-third of their *Mthfr*$^{+/+}$ controls.

Identification of Germ Cell Effects at 6 dpp. Relative testis weights of *Mthfr*$^{-/-}$ pups were normal at 6 dpp, but were severely reduced relative to *Mthfr*$^{+/+}$ males when evaluated at 8 mo of age. We therefore examined the testicular histology at both time points. The germ cell-specific antibody GCNA1 was used to characterize the relative quantity of germ cells in wild-type and MTHFR-deficient males at 6 dpp. GCNA1 is present in all prospermatogonial cells from E11.5 and throughout spermatogonial development until 14 dpp [19]. Staining of *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ testes revealed numerous gonocytes (Fig. 3A), but in the testes of *Mthfr*$^{-/-}$ males there were relatively few gonocytes, regardless of diet; in fact, many cords lacked gonocytes entirely (Fig. 3, B and C).

Given the dramatic reduction in germ cells in *Mthfr*$^{-/-}$ testes, we proposed that germ cell death could account for the observed loss of gonocytes. To examine this possibility, testicular apoptosis was assessed by TUNEL immunostaining at

TABLE 1. Mean body[a] and organ[b] weights of males[c] from experiments 1 and 2.

	Control diet			Betaine-to-weaning (Short-term Betaine)			Betaine beyond weaning (Long-term Betaine)		
	+/+	+/−	−/−	+/+	+/−	−/−	+/+	+/−	−/−
Experiment 1									
6 dpp body	3.93 ± 0.25	2.94 ± 0.88	1.46 ± 0.12[d]	3.65 ± 0.23	3.66 ± 0.20	2.65 ± 0.55[e]	—	—	—
Adult (8 mos) body	31.98 ± 0.87	28.77 ± 1.08	25.47 ± 1.26	31.04 ± 0.68	29.97 ± 1.29	23.68 ± 2.15[f]	—	—	—
Experiment 2									
Adult (3 mos) body	—	—	—	19.68 ± 0.84	21.46 ± 0.61	21.73 ± 1.60	22.59 ± 0.61	21.42 ± 0.93	23.10 ± 0.95
Epididymides	—	—	—	2.44 ± 0.07	2.47 ± 0.06	1.26 ± 0.03[f]	2.43 ± 0.13	2.46 ± 0.08	1.55 ± 0.09[g]
Seminal vesicles	—	—	—	1.68 ± 0.14	1.51 ± 0.13	1.27 ± 0.42	2.16 ± 0.18[f]	1.86 ± 0.11	1.96 ± 0.15[h]

[a] Weight in g (± SE)
[b] Relative weight of paired organs (mg/g body weight) (±SEM).
[c] Adult males in experiments 1 and 2 were 8 and 3 months (mos) old, respectively.
[d] $P < 0.001$ vs. control-diet *Mthfr*$^{+/+}$.
[e] $P < 0.05$ vs. control-diet *Mthfr*$^{-/-}$.
[f] $P < 0.05$ vs. betaine-to-weaning *Mthfr*$^{+/+}$
[g] $P < 0.05$ vs. betaine-beyond-weaning *Mthfr*$^{+/+}$.
[h] $P < 0.05$ vs. betaine-to-weaning *Mthfr*$^{-/-}$.

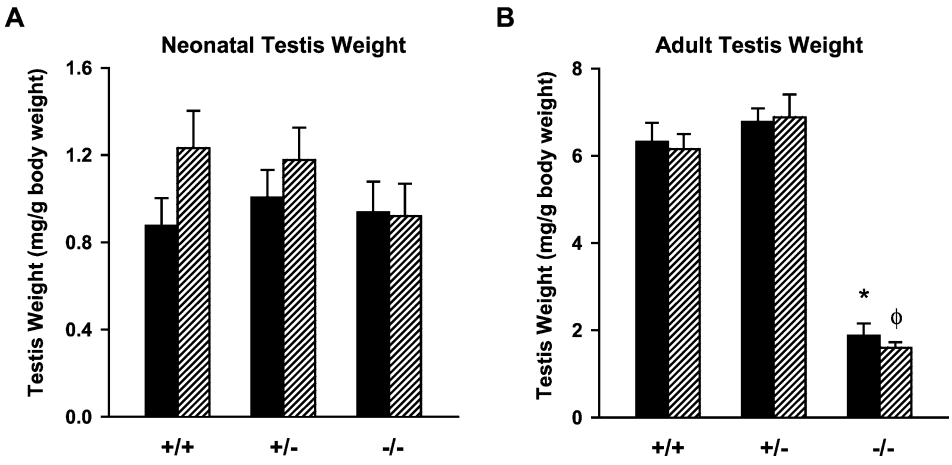

FIG. 2. Effect of MTHFR deficiency and betaine supplementation to weaning (short-term betaine) on testis weights at (**A**) 6 dpp and (**B**) 8 mo of age. Black bars represent males never exposed to supplemental betaine (6 dpp: *Mthfr*$^{+/+}$, n = 4; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 4; 8 mo: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 9; *Mthfr*$^{-/-}$, n = 4); hatched bars represent males provided with supplemental betaine, via their mother (6 dpp: *Mthfr*$^{+/+}$, n = 5; *Mthfr*$^{+/-}$, n = 7; *Mthfr*$^{-/-}$, n =4; 8 mo: *Mthfr*$^{+/+}$, n = 3; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 5). Testis weight is shown relative to body weight. Bars represent means ± SEM. *$P < 0.001$ vs. control-diet *Mthfr*$^{+/+}$; ϕ$P <$ 0.001 vs. betaine-supplemented *Mthfr*$^{+/+}$.

6 dpp. TUNEL-positive cells were quantified and reported as an incidence per 100 tubules (control: *Mthfr*$^{+/+}$, 7.15 ± 2.11; *Mthfr*$^{-/-}$, 31.25 ± 11.93; betaine: *Mthfr*$^{+/+}$, 5.90 ± 2.40; *Mthfr*$^{-/-}$, 4.68 ± 1.33). Apoptotic cells were rare in the testes of unsupplemented and supplemented *Mthfr*$^{+/+}$ males and supplemented *Mthfr*$^{-/-}$ males, but increased significantly ($P < 0.05$) in unsupplemented *Mthfr*$^{-/-}$ males. The increase in apoptotic cells was subtle with only one to two TUNEL-positive cells per tubule, but these cells were consistently observed throughout the whole testis. Although the apoptotic cells were similar in shape and size to germ cells, we cannot be absolutely certain that some were not Sertoli cells.

The increase in apoptosis at 6 dpp in control-diet *Mthfr*$^{-/-}$ males cannot, however, completely account for the low germ cell numbers observed. Although betaine-diet *Mthfr*$^{-/-}$ males had normal levels of apoptosis, these mice had germ cell numbers similar to control-diet *Mthfr*$^{-/-}$ males. We postulated that loss of germ cells in *Mthfr*$^{-/-}$ males may have occurred prenatally. To test this hypothesis, testes of E18.5 male embryos were analyzed

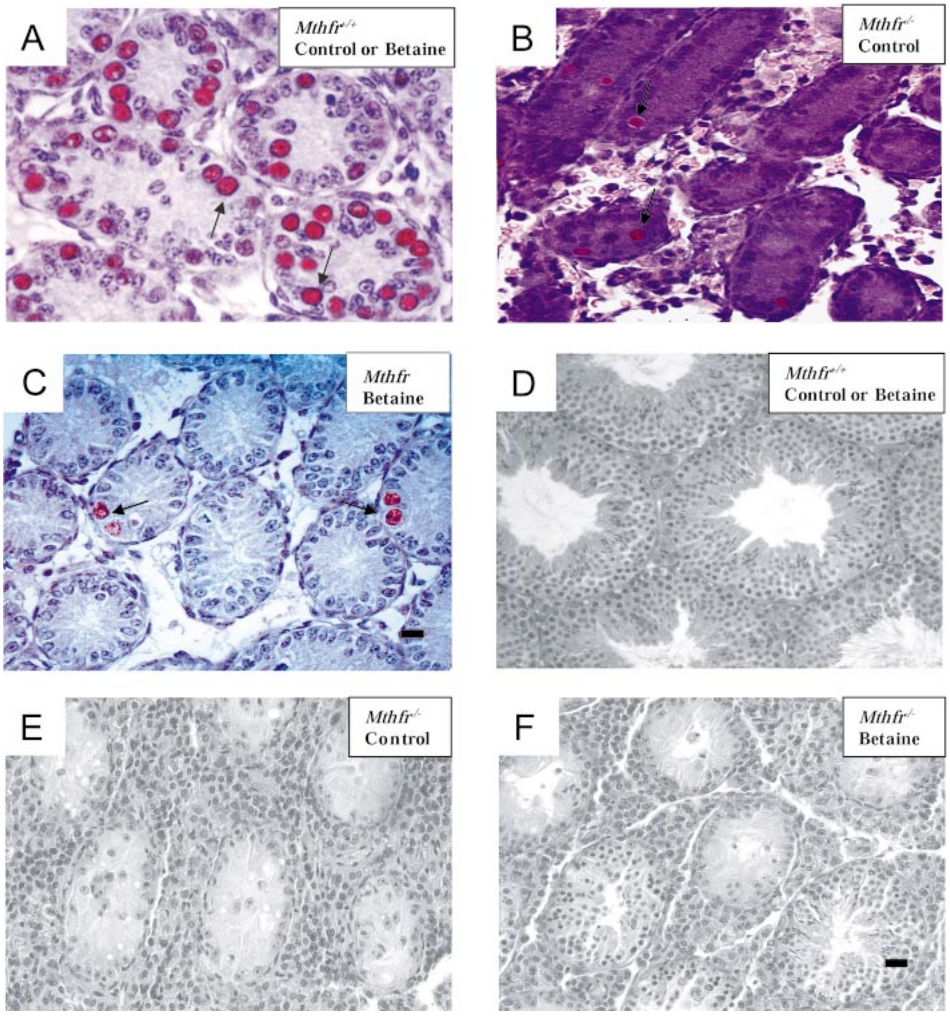

FIG. 3. Short-term betaine: examples of testicular histology at 6 dpp and 8 mo of age. GCNA1 staining at 6 dpp of (**A**) *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ males, with or without betaine supplementation; (**B**) control-diet *Mthfr*$^{-/-}$ males; and (**C**) betaine-supplemented *Mthfr*$^{-/-}$ males. Arrows in (**A**–**C**) indicate germ cells. The bar in (**C**) represents 20 μm for (**A**–**C**). Testicular histology at 8 mo of age in (**D**) *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ males, with or without betaine supplementation to weaning. **E**) Control-diet *Mthfr*$^{-/-}$ males and (**F**) *Mthfr*$^{-/-}$ males provided with betaine supplementation to weaning. The bar in (**F**) represents 50 μm for (**D**–**F**).

quantitatively for germ cell number after immunostaining with GCNA1. Because germ cell numbers appeared similar between the two *Mthfr*$^{-/-}$ dietary groups at 6 dpp, and *Mthfr*$^{-/-}$ pups were difficult to obtain without maternal supplementation, prenatal gonocyte numbers were analyzed only in betaine-supplemented pups. At E18.5, the fetal seminiferous cord is made up of mitotically arrested gonocytes and Sertoli cells. Gonocytes are found in the central area of the cord, and in addition to staining positively for GCNA1, are easily distinguished from Sertoli cells by their large nucleus and several nucleoli, which stain light pink with hematoxylin-eosin. Quantification of GCNA1-stained germ cells revealed similar gonocyte numbers among all three genotypes when analyzed on a per testis (betaine: *Mthfr*$^{+/+}$, 43780 and 35310; *Mthfr*$^{+/-}$, 39615 and 36221; *Mthfr*$^{-/-}$, 44875 and 35310) and per 2000 Sertoli cell basis (betaine: *Mthfr*$^{+/+}$, 687 and 511; *Mthfr*$^{+/-}$, 453 and 487; and *Mthfr*$^{-/-}$, 667 and 411), indicating that gonocyte mitosis is normal before birth and suggesting that the decrease in gonocyte number occurs soon after birth.

Germ cell effects in adult males. Adult testes were analyzed histologically and tubules were scored as abnormal if one or more of the following defects was observed: giant or multinucleate cells, vacuolization, missing germ cell population, or sloughing. *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ testes appeared normal. All spermatogenic stages were present, with normal cellular associations; no differences could be attributed to diet (Fig. 3D). Conversely, all tubules of both control-diet and short-term betaine treated *Mthfr*$^{-/-}$ males were abnormal, and an average of 80% to 90% of tubules within each group appeared to lack germ cells (Fig. 3, E and F). Where possible tubules were staged (<10%); however, the lack of elongating spermatids, frequent appearance of degenerating pachytene spermatocytes, and the disruptive effects of pervasive vacuolization made staging quite difficult and inexact. In tubules that could be staged, germ cell associations were appropriate, but tubules were often missing one or more germ cell populations, had extensive vacuolization, or exhibited sloughing. All stages appeared to be equally affected in *Mthfr*$^{-/-}$ males, regardless of diet, and specific abnormalities did not correspond to particular stages. In those tubules that had germ cells yet could not be staged, abnormalities were similar to those in staged tubules, but in these cases they disrupted the germ cell arrangement to such an extent that staging was not possible. Round spermatids were generally the most mature germ cell population; elongating spermatids were observed in less than 2% of tubules.

The tubules of individual MTHFR-deficient males were varied in appearance such that the timing of the MTHFR defect within spermatogenesis was difficult to pinpoint. Some tubules lacked germ cells entirely, whereas others had pachytene spermatocytes, but not germ cells that were more mature. Still other tubules, although more rare, exhibited elongating spermatids, but had considerable vacuolization. Spermatogenesis did not appear to halt at any one particular stage or point during germ cell development. Not one particular germ cell population was affected, and when vacuolization was present, it was not limited to one part of the seminiferous epithelium.

There was considerable variation among males within each *Mthfr*$^{-/-}$ treatment group. Of the three control-diet *Mthfr*$^{-/-}$ males, one had almost no germ cells and the vast majority of tubules appeared to contain only Sertoli cells; another male had germ cells in 34% of tubules, the majority of which could be staged, while the remaining tubules lacked germ cells; and the third male (shown in Fig. 3E) had only a few tubules that could be staged, and in 94% of tubules, germ cells were absent. In those few tubules with germ cell differentiation, sloughing was often evident, as was vacuolization.

At 8 mo, testicular histology of betaine-supplemented *Mthfr*$^{-/-}$ males was similar to control-diet *Mthfr*$^{-/-}$ males, with the exception that elongated spermatids were seen more frequently, in an average of 10% of tubules (Fig. 3, E and F). Variability was also prevalent in the supplemented *Mthfr*$^{-/-}$ males. Three of five males had almost no germ cells and had histology resembling that shown in Figure 3E, but approximately 20% of the tubules in the remaining two males could be staged (Fig. 3F). As with unsupplemented *Mthfr*$^{-/-}$ males, abnormalities were predominantly vacuolization, sloughing, and missing germ cell populations, and did not vary by stage.

With the decrease in germ cells was an apparent increase in the interstitial cell population in both nullizygous groups. This may be a visual consequence of decreased germ cell numbers and thus decreased tubule diameter.

Betaine Supplementation Beyond Weaning and MTHFR Deficiency (Experiment 2: Short-Term Versus Long-Term Betaine)

Effects on male reproductive organ weights. In the first experiment, betaine-supplemented *Mthfr*$^{-/-}$ males exhibited decreased apoptosis at 6 dpp, but once weaned from this diet, germ cell development became abnormal to nonexistent. In the second experiment, we therefore considered the positive effects that continued supplementation of betaine, beyond weaning and throughout adulthood, might have on spermatogenesis in *Mthfr*$^{-/-}$ males.

Unlike the first experiment, there were no deviations in body weight among genotypes or diets (Table 1). Additionally, spleen weight, white blood cell count, and hemoglobin level did not change with respect to genotype or diet (data not shown). However, upon examination of the male reproductive system, several effects became apparent.

Relative epididymal weights (Table 1) were severely decreased in both *Mthfr*$^{-/-}$ groups, although this reduction was somewhat remedied, but not significantly, by the continuation of betaine beyond weaning. Similarly, genotype had a dramatic effect on testis weight, although as seen throughout both experiments, there was no observed heterozygote effect. Testis weights of *Mthfr*$^{-/-}$ males were severely reduced in both dietary groups, yet the mean relative testis weight of long-term betaine *Mthfr*$^{-/-}$ males was twofold higher than those given betaine only until weaning (Fig. 4). The 76% decrease in the testis weight of *Mthfr*$^{-/-}$ males, relative to *Mthfr*$^{+/+}$ males, was similar to that seen in the first experiment. This decrease was slightly, although not significantly, alleviated by the addition of betaine postweaning; in these males the decrease in relative testis weight was only 60% (Fig. 4).

One possible explanation for changes in testicular weight is altered testosterone levels. The seminal vesicles are accessory sex organs sensitive to changes in androgen status; weights of these organs often indicate changes in testosterone. Long-term betaine supplementation resulted in significantly higher seminal vesicle weights in both *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ males when compared with males of the same genotype provided with betaine only until weaning (Table 1). Within the same diet, however seminal vesicle weights were similar across genotypes, suggesting that the decrease

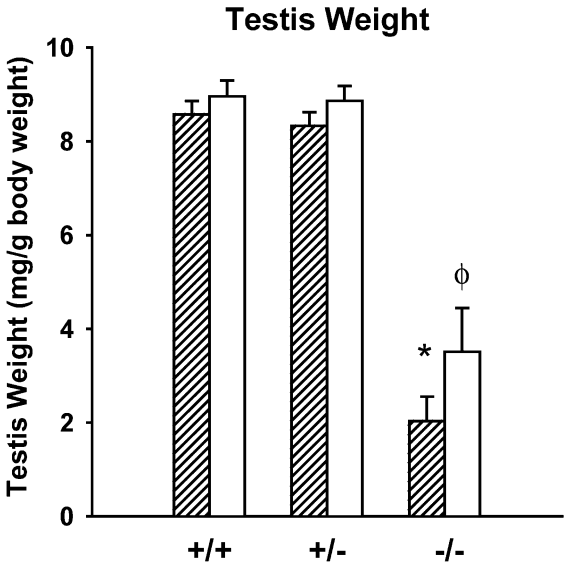

FIG. 4. Effect of MTHFR deficiency and long-term betaine supplementation on relative testis weight (shown per gram body weight). Hatched bars represent males given betaine only to weaning (*Mthfr*$^{+/+}$, n = 8; *Mthfr*$^{+/-}$, n = 11; *Mthfr*$^{-/-}$, n = 3) and white bars represent males provided with lifelong betaine supplementation (preweaning and postweaning; *Mthfr*$^{+/+}$, n = 8; *Mthfr*$^{+/-}$, n = 7; *Mthfr*$^{-/-}$, n = 5). Bars represent means ± SEM. *$P < 0.001$ vs. control-diet *Mthfr*$^{+/+}$; ϕ$P < 0.001$ vs. betaine-diet *Mthfr*$^{+/+}$.

in *Mthfr*$^{-/-}$ testis weights relative to those of *Mthfr*$^{+/+}$ males were not the result of altered testosterone levels.

Positive effects of betaine on spermatogenesis. *Mthfr*$^{-/-}$ males provided with short-term betaine in this study displayed testicular abnormalities identical to those observed in adults of the first study (Figs. 3F and 5, A and B). There was again considerable variation among *Mthfr*$^{-/-}$ members of the dietary groups. All tubules of one male appeared to lack germ cells (Fig. 5A), while germ cell development was evident in 14% and 76% of tubules in the other two males. The majority of tubules with germ cells could be staged, but all tubules were scored as abnormal because of pervasive vacuolization, sloughing, and missing germ cell populations. Of note, considerably more tubules could be staged in this *Mthfr*$^{-/-}$ group compared with staging in the first study. This increase, however, was because in one male (Fig. 5B), nearly 76% of tubules could be staged; the remaining males had levels similar to the first experiment. As in the first experiment, those tubules that could be staged contained normal germ cell associations, although vacuolization, multinucleate cells, and sloughing were generally evident and, frequently, one or more germ cell populations were missing. Defects were not typical to one stage, and there was no relationship between the tubule stage and missing germ cell population. Elongating spermatids were observed in an average of less than 10% of tubules.

Remarkably, while some tubules of *Mthfr*$^{-/-}$ males supplemented with long-term betaine resembled those of males

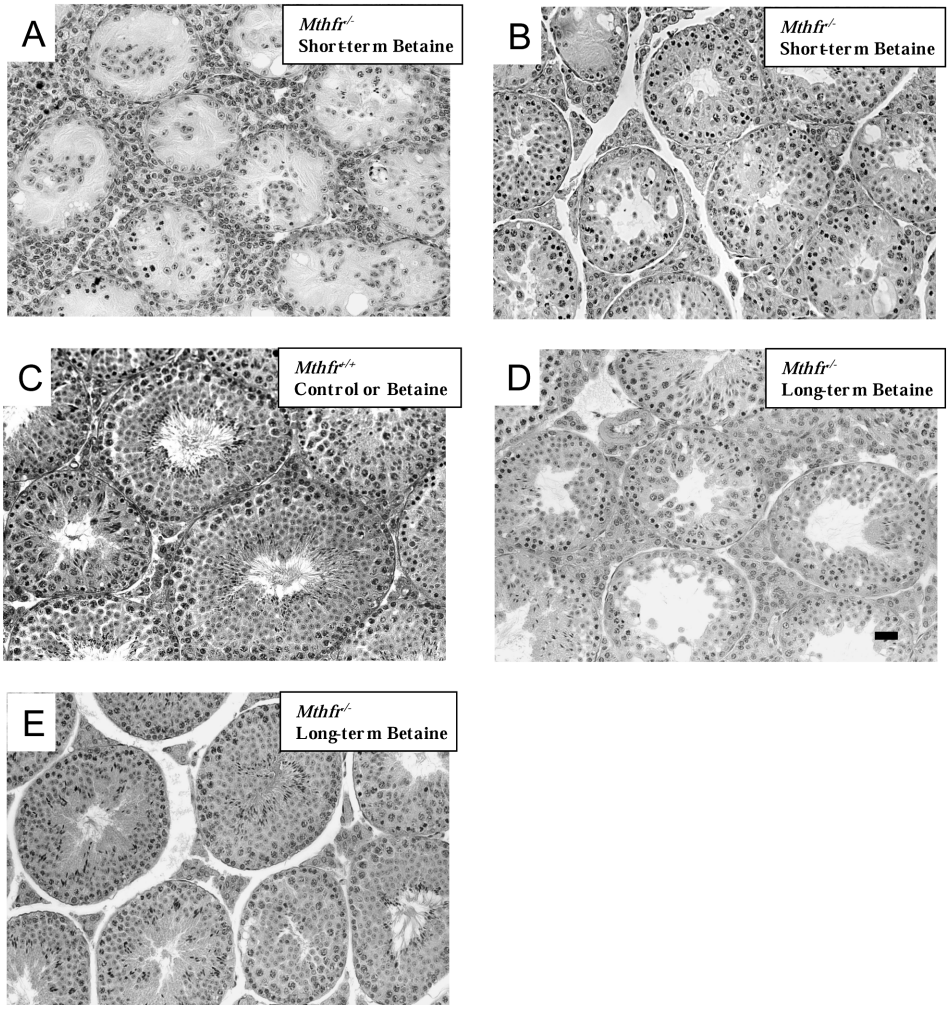

FIG. 5. Long-term betaine: examples of testicular histology at 3 mo of age. **A**, **B**) Variability in testicular histology observed in *Mthfr*$^{-/-}$ males exposed to betaine only until weaning. **C**) *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ males with betaine supplementation to weaning, and lifelong betaine supplementation. **D**, **E**) Variability in testicular histology observed in *Mthfr*$^{-/-}$ males provided with betaine up to and beyond weaning. In *Mthfr*$^{-/-}$ males receiving long-term betaine, approximately 28% of all tubules (mean of all males within the group) display normal spermatogenesis. The bar in (**D**) indicates 50 μm for (**A**–**E**).

who received betaine only to weaning (Fig. 5D), the majority displayed much improved spermatogenesis (Fig. 5E). Indeed, quantification of abnormal tubules—those tubules with multinucleate/giant cells, degenerating germ cells, sloughing of immature germ cells, or lacking one or more germ cell populations—revealed that all (100% ± 0%) tubules in *Mthfr*$^{-/-}$ males given betaine only to weaning were abnormal, however, significantly fewer tubules (71% ± 12%) from *Mthfr*$^{-/-}$ males given long-term betaine were abnormal. Normal spermatogenesis was present in the remaining tubules of *Mthfr*$^{-/-}$ males given long-term betaine and elongating spermatids were observed, on average, in more than 25% of tubules (Fig. 5E). As with the other dietary *Mthfr*$^{-/-}$ groups, there was considerable variation within the long-term betaine *Mthfr*$^{-/-}$ group. Four of the five males within this group exhibited normal spermatogenesis, but to differing extents (percent normal tubules: 14%, 29%, 32%, 67%). Testicular histology of the male with 14% normal spermatogenesis is shown in Figure 5D; Figure 5E is an example of the histology from the male with 67% normal spermatogenesis. Despite provision of long-term betaine, one *Mthfr*$^{-/-}$ male had no normal tubules and histology similar to that of Figure 5B. Again, similar defects were observed across all stages and when abnormal and normal tubules were staged, a comparable stage distribution was evident in both normal and abnormal tubules.

Effects on testicular sperm production. Mean testicular sperm count per gram testis weight was severely decreased in *Mthfr*$^{-/-}$ males given betaine only to weaning and was only 6% of genotypic controls (Fig. 6A). Betaine treatment beyond weaning resulted in higher sperm production, per gram testis weight, in *Mthfr*$^{-/-}$ males. Although still significantly lower than their genotypic controls, this mean relative sperm production was a significant improvement over sperm counts in *Mthfr*$^{-/-}$ males given betaine to weaning ($P < 0.01$).

MTHFR and fertility. Because of the observed improvements in testicular histology and sperm numbers, we postulated that betaine supplementation into adulthood would improve *Mthfr*$^{-/-}$ male fertility. Males from each group were mated over a period of 2 wk with four females, and mating success was evident by the presence of a vaginal plug. All data were calculated and are shown on a per male basis. Regardless of genotype or diet, mating performance was similar for all groups (data not shown). Fertility in *Mthfr*$^{-/-}$ males given short-term betaine, however, was completely compromised. Pregnancy rate, calculated as the percent plug-positive females that gave birth, was zero in females mated to males given betaine only until weaning (Fig. 6B). In contrast, two of the five *Mthfr*$^{-/-}$ males given long-term betaine were capable of siring offspring, a fertility rate significantly higher ($P < 0.05$) than in the *Mthfr*$^{-/-}$ group given betaine only to weaning. One male given long-term betaine sired

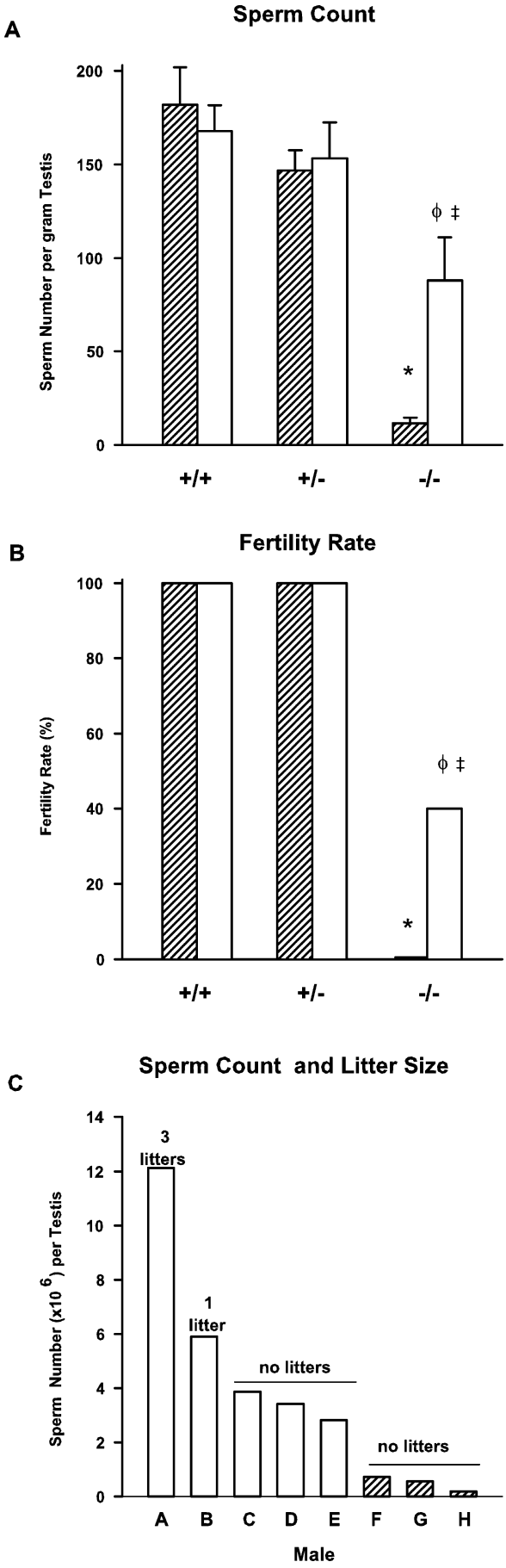

FIG. 6. Effect of MTHFR deficiency and long-term betaine supplementation on (**A**) testicular sperm counts, per gram testis weight, and (**B**) male fertility. **C**) Relationship between total sperm counts of *Mthfr*$^{-/-}$ males with or without betaine postweaning and the number of litters sired. Hatched bars represent males given betaine only to weaning (for sperm counts: *Mthfr*$^{+/+}$, n = 3; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 3) (for matings: *Mthfr*$^{+/+}$, n = 8; *Mthfr*$^{+/-}$, n = 11; *Mthfr*$^{-/-}$, n = 3) and white bars represent males provided with lifelong betaine supplementation (preweaning and postweaning) (for sperm counts: *Mthfr*$^{+/+}$, n = 3; *Mthfr*$^{+/-}$, n = 3; *Mthfr*$^{-/-}$, n = 5) (for matings: *Mthfr*$^{+/+}$, n = 8; *Mthfr*$^{+/-}$, n = 7; *Mthfr*$^{-/-}$, n = 5). Bars represent means ± SEM. *$P < 0.001$ vs. control-diet *Mthfr*$^{+/+}$; ϕ$P < 0.001$ vs. betaine-diet *Mthfr*$^{+/+}$; ‡$P < 0.001$ vs. control-diet *Mthfr*$^{-/-}$.

three litters and another male, one litter (Fig. 6C). When litters were analyzed for pup number and sex ratio there were no differences among genotypes or diets (data not shown).

Only two of the five *Mthfr*$^{-/-}$ males provided with the long-term betaine were able to sire litters, even though relative sperm counts were increased in all these males compared with *Mthfr*$^{-/-}$ males given betaine only until weaning. Moreover, there was no relationship between an individual's relative testis weight or relative sperm count and fertility. Total sperm production, however, was severely decreased in those males that were infertile (Fig. 6C). As illustrated in Figure 6C, the *Mthfr*$^{-/-}$ male that sired three litters had a total sperm production that was twice that of the *Mthfr*$^{-/-}$ male that sired one litter. Also of note is that infertile *Mthfr*$^{-/-}$ males on long-term betaine had total sperm counts that were at least three and one-half times the counts of *Mthfr*$^{-/-}$ males given betaine only until weaning. There appears to be a threshold of sperm production in MTHFR-deficient males, below which males are infertile. Male C had a total sperm count that was only 35% less than Male B, yet was unable to sire any offspring. Sperm production did not increase correspondingly with increased testicular weight. Relative testis weights were similar for some males given long-term betaine and males given short-term betaine. Despite this, those *Mthfr*$^{-/-}$ males given long-term betaine each had total sperm production that was at least three and one-half times higher than *Mthfr*$^{-/-}$ mice given betaine only to weaning, suggesting that although testis weight may not increase significantly with continued betaine supplementation, the ability to maintain spermatogenesis increases with prolonged betaine supplementation.

DISCUSSION

MTHFR and Spermatogenesis

The association between mutations/polymorphisms in MTHFR and other folate pathway genes and decreased fertility or pregnancy success in females has been exhaustively examined (see [24] for a review). However, the role that MTHFR may play in male fertility has not yet been examined in detail. Here we show for the first time that male mice lacking MTHFR suffer severe reproductive consequences; spermatogenesis in these mice fails during early postnatal development and results in complete infertility.

In the original study of the MTHFR knockout [4], mice were on a mixed genetic background and *Mthfr*$^{-/-}$ males were reported to be fertile. However, our study indicates that after multiple generations of backcrossing into the BALB/c strain, MTHFR deficiency results in male infertility, with extensive testicular histological abnormalities. Presumably, the mixed genetic background of these early generations provided a degree of hybrid vigor, resulting in less deleterious effects of the MTHFR mutation. Although Chen et al. [4] and others [14] have described a heterozygous phenotype in MTHFR-deficient mice, there was no observed heterozygote effect in our studies of male reproduction.

Neonatal Testis

Despite normal relative testis weights, there were significant histological differences in seminiferous cord morphology at 6 dpp between *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ mice. *Mthfr*$^{-/-}$ males had extremely reduced gonocyte populations and in unsupplemented *Mthfr*$^{-/-}$ males, displayed significantly increased levels of apoptosis. At 6 dpp the seminiferous cords normally contain only Sertoli cells and gonocytes, with the latter comprising only 16% of the total cellular content of the tubules [25]. Because gonocytes make up such a small portion of the cellular population, even a substantial reduction in germ cells would likely not manifest as a significant decrease in testis weight. Prior to birth, gonocytes stop proliferating [26, 27] and do not resume replication until about 1.5 dpp. One possible explanation for the dramatically reduced gonocyte numbers at 6 dpp is that some gonocytes in *Mthfr*$^{-/-}$ males fail to resume mitosis postnatally. It has been suggested that maternal folate pools may protect *Mthfr*$^{-/-}$ pups throughout gestation, but once born, these mice are unable to meet the increased need for methyl groups that elevated cellular proliferation demands [14]. However, if failure to proliferate postnatally were the only cause for declining germ cell numbers, we would expect similar numbers of gonocytes at E18.5 and 6 dpp. That there is a dramatic reduction in gonocyte number between E18.5 and 6 dpp suggests that there is likely another reason, perhaps in addition to nonproliferation, for the reduced gonocyte numbers observed at 6 dpp in *Mthfr*$^{-/-}$ males.

Shortly after birth, gonocytes begin relocating from the central area of the seminiferous cord, where the lumen later develops, to the basement membrane [26, 28]. Relocation is typically complete by 6 dpp [26]. Relocation to the periphery of the tubule is not necessary for reproliferation and vice versa [29–31], however it has been postulated that gonocytes failing to undergo relocation apoptose or are removed with the formation of the lumen [32, 33]; hence, movement to the cord periphery may be necessary to avoid apoptosis or phagocytosis by Sertoli cells [32]. In our study, apoptotic cells were typically located toward the central area of the cord. We suggest that the reduced germ cell number seen at 6 dpp and in adults may be the result of apoptosis of gonocytes that fail to replicate postnatally or are improperly aligned along the basal aspects of the seminiferous tubule. Our results indicate that such an event occurred sometime before 6 dpp and may have occurred soon after birth, when relocation and reproliferation are initiated (2–4 dpp). Unfortunately, the difficulty of obtaining *Mthfr*$^{-/-}$ pups without betaine supplementation makes studies of these early postnatal days very difficult.

Adult Testis

Mthfr$^{-/-}$ males exhibited extremely abnormal testicular histology, with tubules that were nearly devoid of germ cells, and rarely were the more mature germ cell populations observed. The exact nature and timing of the MTHFR defect within spermatogenesis is unclear. Spermatogenesis did not terminate at any one specific stage or point in development and it was not one particular stage, germ cell population, or layer of the seminiferous epithelium that was affected by MTHFR deficiency. Also striking was the degree of heterogeneity among *Mthfr*$^{-/-}$ males of the same diet. Furthermore, it is unclear at this time whether the testicular defect seen with MTHFR deficiency is due to abnormalities within the somatic cells of the testis or the germ cells themselves. Spermatogonial stem cell transplantation from *Mthfr*$^{-/-}$ males into germ-cell deficient mice or the use of *Mthfr*$^{-/-}$ males as the recipients of stem cells from normal mice would aid in this discrimination. This method has been used previously to localize defects within the testis [34] and could be used to great advantage here to examine the role of MTHFR in testicular development.

Mthfr$^{-/-}$ males appeared to have greatly expanded populations of interstitial cells. Increased Leydig cell proliferation could result from a rise in leuteinizing hormone. However, an increase in Leydig cells would likely manifest as an increase in testosterone. That seminal vesicle weights, a well-accepted proxy for monitoring changes in testosterone, were similar across genotypes within the same diet suggests that the apparent increase in interstitial cells may simply be an optical illusion, the result of decreased germ cell numbers and a concomitant reduction in tubule diameter.

MTHFR is crucial to the folate pathway and disruption of this enzyme compromises mouse development in several ways. Schwahn and colleagues [14] postulated that accumulation of homocysteine and impaired transmethylation reactions (Fig. 1) may be primary factors in MTHFR pathology. These factors may also underlie the spermatogenic defects observed with MTHFR deficiency. MTHFR deficiency is believed to impede the methylation of a wide variety of substrates, including proteins, DNA, RNA, and histones through decreased methionine supply. This condition is potentially exacerbated by accumulation of SAH, which can directly inhibit methylation reactions through strong binding of SAM-dependent methyltransferases [35]. One transmethylation reaction, DNA methylation, has been shown to be crucial for normal spermatogenesis [23, 36] and thus may be particularly vulnerable to the changes in methyl pools brought about by deficiency in MTHFR. Moreover, inactivation of MTHFR can also disrupt cellular nucleotide pools, effectively enhancing cellular proliferation. If transmethylation processes are not able to keep pace with increased cell division, diminished cell survival may result.

Betaine and Spermatogenesis

In severe MTHFR deficiency, provision of a methyl donor can restore remethylation of homocysteine to methionine [18]. As such, betaine has proven clinically useful in treating MTHFR deficiency [16, 37, 38]. Betaine is the substrate for BHMT (Fig. 1), which typically acts in concert with methionine synthetase to keep homocysteine levels in check via remethylation of this amino acid [17, 39]. Because administration of a 2% oral betaine solution to females during mating, pregnancy, and nursing results in a fourfold increase in pup survival and improved liver and brain phenotypes [14], we postulated that betaine supplementation might also improve spermatogenesis in *Mthfr*$^{-/-}$ males.

In rodents, BHMT activity dramatically increases soon after birth, indicating a pronounced need for this enzyme [40], perhaps because of increased cellular proliferation at this time. Homocysteine remethylation is responsible for elimination of 50% of homocysteine levels; and this remethylation is equally divided between the BHMT pathway and 5-methyltetrahydrofolate, provided by MTHFR [41]. Under typical dietary conditions, remethylation provides more than half of normal methionine requirements [42]. In MTHFR-deficient mice, there is an increased reliance on remethylation via the BHMT pathway, perhaps because of lowered methionine availability [14, 43–45]. Although gonocyte number was not different in control-diet and betaine-diet 6 dpp *Mthfr*$^{-/-}$ males, there was some improvement as indicated by decreased levels of apoptosis. Because *Mthfr*$^{-/-}$ pups with betaine supplementation have significantly reduced homocysteine levels [14], they may therefore be able to maintain, to a limited extent, the higher levels of transmethylation that increased cellular proliferation, soon after birth, requires. As such, betaine supplementation may help these mice maintain a minimal proliferating gonocyte population that may be spared from apoptosis. Hence, provision of betaine before weaning may help to maintain a gonocyte population that can later populate the testis. The precise mechanism by which betaine improves spermatogenesis is unclear. However, although the localization of BHMT has not been examined in the gonads [46, 47], members of the solute carrier (SLC) 6 family, responsible for betaine transport have been identified within the testis [48]. Alternatively, higher circulating levels of SAM, observed with betaine supplementation [14], may be taken up by the testis and thus enable transmethylation reactions to continue within the testis.

It is clear from our studies though, that any benefits derived from early betaine exposure did not carry through to adulthood if betaine was removed at weaning. Adult *Mthfr*$^{-/-}$ males given betaine to weaning exhibited a testicular phenotype similar to control-diet *Mthfr*$^{-/-}$ males. Decreased apoptosis at 6 dpp, however, raised the question of whether continued betaine administration to *Mthfr*$^{-/-}$ males, beyond weaning, might have positive effects on testis development and spermatogenesis. Indeed, with long-term betaine provision came improved testis weights, histology, and remarkably, a small but significant increase in fertility.

Our most intriguing finding was that fertility, in a subset of *Mthfr*$^{-/-}$ males, could be restored upon provision of long-term betaine. Fertility appeared to be related to overall testicular sperm production. Thus, we have produced an interesting model for further studies of dietary/nutritional amelioration of genetic infertility. That *Mthfr*$^{-/-}$ males given betaine only until weaning had sperm but were infertile suggests that there is some intrinsic defect with sperm produced in MTHFR-deficient mice. Robaire et al. [49] have shown that sperm production must be severely reduced to less than 5% of normal to have an effect on fertility. Even in long-term betaine-supplemented *Mthfr*$^{-/-}$ males, only two of the five were able to sire litters. A threshold above which a minimum number of normal sperm are produced must exist. However, it remains to be seen whether betaine is constantly required, from gestation through adulthood, to promote spermatogenesis, or if it could be used acutely in adults to renew spermatogenesis. Such acute treatments have been shown to partially rescue other defects of MTHFR deficiency [18]. It is possible that betaine may be necessary only until the testis is fully developed. Future studies are needed to determine the interval and timing of betaine supplementation necessary to recoup male fertility.

That betaine supplementation did not result in complete amelioration of the testicular phenotype of *Mthfr*$^{-/-}$ males agrees with the finding by Schwahn et al. [14], who demonstrated that increased betaine corrected some characteristics of severe MTHFR deficiency but not to wild-type levels. Although homocysteine and its metabolite, SAH, were significantly decreased in betaine-supplemented *Mthfr*$^{-/-}$ mice, these levels were still substantially higher than those in *Mthfr*$^{+/+}$. These amounts of homocysteine and SAH may be adequate to inhibit transmethylation reactions. Moreover, correction did not occur to the same extent in all males provided with long-term betaine. It is currently unknown why some males would respond to such a high degree to treatment while others would not. Perhaps variations in neonatal or early postnatal homocysteine or meth-

ylation levels may explain the degree of response to betaine supplementation and the variation seen later in life.

Implications

Our finding that lack of MTHFR impairs spermatogenesis provides further support for the hypothesis that 677C→T mutation in MTHFR may explain some degree of human male infertility. We have shown a substantial improvement in spermatogenesis in *Mthfr*$^{-/-}$ mice with continual provision of dietary betaine. Our findings suggest that infertile men with MTHFR mutations may benefit from diets supplemented with alternative methyl donors. Indeed, infertile men provided with folic acid report improved fertility and decreased round cell azoospermia [50]; such men may have defects in MTHFR or other enzymes involved in the folate pathway enzymes.

Many other mutations have been identified within the human *MTHFR* gene, among these, a common A1298T variant. The decrease in enzyme activity associated with the C677T polymorphism is greater than that observed with the 1298 variant. Other mutations in the folate pathway enzymes may have roles in hampering male fertility. Similar to the *Mthfr*$^{-/-}$ model, however, spermatogenesis is extremely difficult to study in these models due to complex systemic effects. Inactivation of these enzymes in a testis-specific manner may help to elucidate the role of these enzymes in testicular health.

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