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Investigating the effects of dietary folic acid on sperm count, DNA damage and mutation in Balb/c mice

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ABSTRACT

To date, fewer than 50 mutagens have been studied for their ability to cause heritable mutations. The majority of those studied are classical mutagens like radiation and anti-cancer drugs. Very little is known about the dietary variables influencing germline mutation rates. Folate is essential for DNA synthesis and methylation and can impact chromatin structure. We therefore determined the effects of folic acid-deficient (0 mg/kg), control (2 mg/kg) and supplemented (6 mg/kg) diets in early development and during lactation or post-weaning on mutation rates and chromatin quality in sperm of adult male Balb/c mice. The sperm chromatin structure assay and mutation frequencies at expanded simple tandem repeats (ESTRs) were used to evaluate germline DNA integrity. Treatment of a subset of mice fed the control diet with the mutagen ethylnitrosourea (ENU) at 8 weeks of age was included as a positive control. ENU treated mice exhibited decreased cauda sperm counts, increased DNA fragmentation and increased ESTR mutation frequencies relative to non-ENU treated mice fed the control diet. Male mice weaned to the folic acid deficient diet had decreased cauda sperm numbers, increased DNA fragmentation index, and increased ESTR mutation frequency. Folic acid deficiency in early development did not lead to changes in sperm counts or chromatin integrity in adult mice. Folic acid supplementation in early development or post-weaning did not affect germ cell measures. Therefore, adequate folic acid intake in adulthood is important for preventing chromatin damage and mutation in the male germline. Folic acid supplementation at the level achieved in this study does not improve nor is it detrimental to male germline chromatin integrity.

1. Introduction

Germline mutation may lead to various detrimental outcomes including embryonic lethality, inherited genetic disease or trans-generational genetic instability [1]. Since the germline has a very low spontaneous mutation frequency, germ cell mutation assays generally require large numbers of animals and high chemical doses to establish an agent as mutagenic [2]. As a result, much less is known about the environmental variables that influence germ cell mutation rates compared to somatic cells. To date, fewer than 50 mutagens have been studied for their ability to cause heritable mutations [3]. Existing evidence supports the germline mutagenicity of various agents including radiation, mutagenic anti-cancer drugs, and specific environmental mixtures (tobacco smoke, urban air pollution) [4–11]. However, relatively little is known about dietary variables that affect germ cell mutation rates.

Folate, an essential B vitamin, is required for the de novo synthesis of purines, thymidylate and methionine [12]. As such, folate is required for the maintenance of DNA synthesis and methylation, and consequently chromatin structure and gene expression (Fig. 1). In somatic cells, folate deficiency can lead to increased uracil incorporation into DNA, DNA double strand breaks, genome instability and DNA hypomethylation [13–17]. However, few studies have examined the effects of folic acid deficiency on germ cells.

Folate deficiency is associated with indicators of reduced male fertility. For example, low folate in seminal plasma is correlated with decreased sperm counts and increased sperm DNA damage in humans [18,19]. An inverse relationship between total daily folate intake and the frequency of aneuploid sperm in humans has also been observed [20]. In mice, treatment with methotrexate,

Abbreviations: DFI, DNA fragmentation index; ENU, ethylnitrosourea; ESTR, expanded simple tandem repeat; RBC, red blood cell; SCSA, sperm chromatin structure assay; SDS, sodium dodecyl sulfate.

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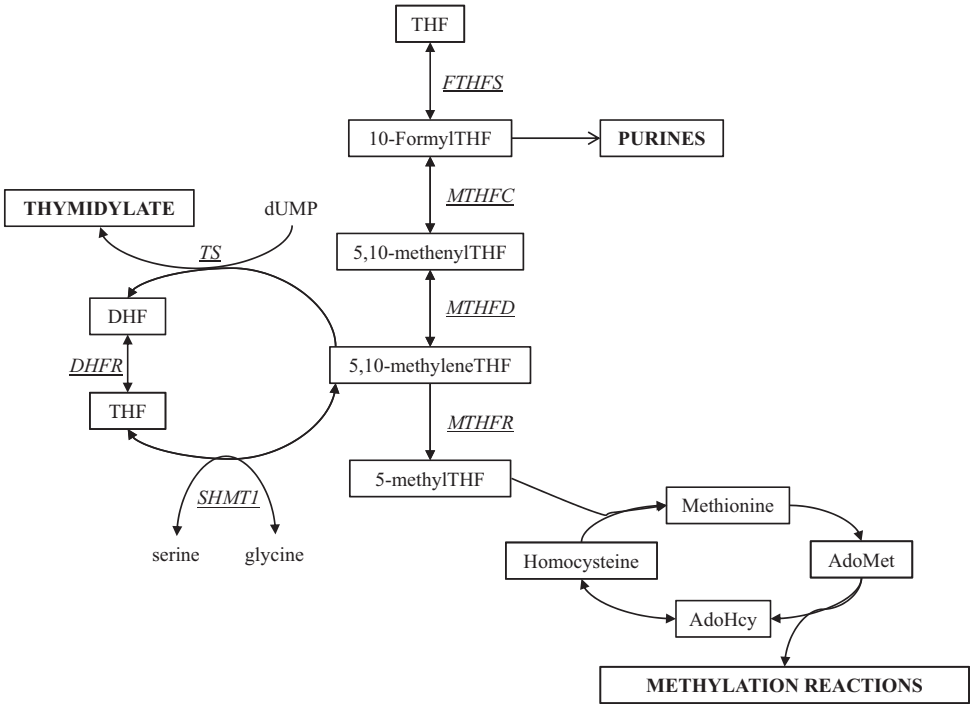

Fig. 1. Folate-mediated one-carbon metabolism. Folate is required for the synthesis of purines, thymidylate and methionine. Methionine can be converted to AdoMet, the major methyl donor used for cellular methylation reactions. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; FTHFS, formyltetrahydrofolate synthetase; MTHFC, methenyltetrahydrofolate cyclohydrolase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; SHMT1, cytoplasmic serine hydroxymethyltransferase; THF, tetrahydrofolate, TS, thymidylate synthase.

an antifolate chemotherapeutic, leads to decreased sperm counts and increased sperm DNA damage [21,22]. These studies demonstrate that folate impacts sperm health and DNA integrity, suggesting that folate deficiency may be mutagenic to germ cell DNA.

In 1998, Canada and the US mandated fortification of white flour with folic acid to reduce the incidence of neural tube defects [23]. Folic acid fortification has successfully eliminated folate deficiency in Canada; however, the folate status of a significant proportion of the general population is indicative of folic acid intakes at or above the tolerable upper intake level [24,25]. High folic acid intake can overwhelm enterocyte metabolism and result in the presence of unmetabolized folic acid in circulation [26,27]. Circulating folic acid must be metabolized by the tissues and the presence of unmetabolized folic acid could result in the accumulation of cellular dihydrofolate, an inhibitor of methylenetetrahydrofolate reductase [28] and thymidylate synthase [29]. Inhibition of these enzymes has been proposed to decrease methionine and thymidylate synthesis [30]. Consequently, high folic acid intake may, paradoxically, result in a functional folate deficiency, resulting in increased mutation rates and genome instability.

In the present study we explore the effects of folic acid deficiency and supplementation on male germ cell integrity. We evaluated three measures of sperm health including sperm count, DNA damage using the sperm chromatin structure assay (SCSA), and DNA sequence mutations at tandemly repeated sequences. The effects of dietary folic acid in utero and during lactation (early development) or from weaning for 15 weeks were investigated. Three dietary groups representing folic acid deficiency (0 mg/kg), the recommended daily intake of folic acid (control, 2 mg/kg), and folic acid supplementation (6 mg/kg) at three times the daily recommended intake and approximating the consumption of a 1 mg/day folic acid supplement and folic acid from fortified foods were used.

2. Materials and methods

2.1. Mice

All mice were cared for in accordance with the Guidelines of the Canadian Council on Animal Care, described in the CACC Guide to the Care and Use of Experimental Animals [31]. The study was approved by the Health Canada Animal Care Committee.

2.1.1. Diets

The control diet was AIN-93G [32], which contains 2 mg folic acid/kg diet (Dyets, Inc., Bethlehem, PA). The folic acid deficient diet was modified AIN-93G containing 0 mg folic acid/kg diet (Dyets, Inc.) and the folic acid supplemented diet was modified AIN-93G containing 6 mg folic acid/kg diet (Dyets, Inc.). All diets contained the same levels of choline and methionine.

2.1.2. Colony founders

52 Female and 26 male Balb/c mice (7 weeks old) were purchased from Jackson Labs (Bar Harbor, ME) to establish a breeding colony and to give rise to the F0 generation. At 8 weeks of age breeding trios were set-up with 2 females and 1 male, all were fed a fixed formula, non-purified diet (Teklad Diets, Madison, WI) during the breeding and post-natal periods.

2.1.3. F0

At 21 days of age, groups of female mice were weaned to one of the three experimental diets, deficient ($n = 32$), control ($n = 32$) or supplemented ($n = 32$) (Fig. 2). At 7 weeks of age, breeding trios were set-up with aged-matched male mice that had been fed the control diet, and fed the diet to which the female had been weaned. Female mice were fed their respective diets during pregnancy, postpartum and lactation.

2.1.4. F1

At 21 days of age, 12 male mice from separate litters were selected from the deficient and the supplemented maternal diet groups (12 pups from each group) and were weaned to the control folic acid diet (2 mg/kg). Twenty-four male mice from the control maternal diet group (two per independent litter) were weaned to the control diet. Male mice from dams fed the control diet were also weaned to either the deficient or supplemented diets ($n = 12$ for each diet from 12 independent litters). Male mice were fed their respective diets for 15 weeks and killed at 18 weeks of age (Fig. 2). For breeding purposes (F2 and F3 generations were produced as part of another study) all mice were fed the control diet for 3 weeks prior to sacrifice to ensure that females, with which the males were co-housed, were folate replete.

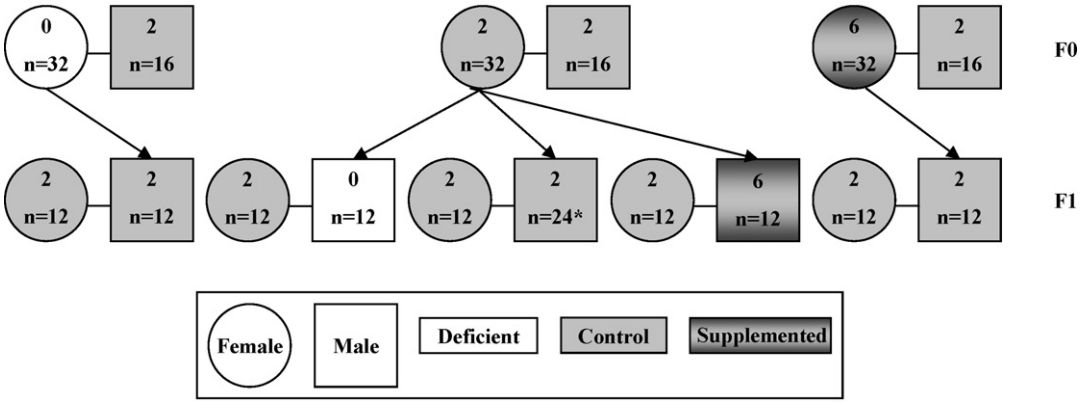

Fig. 2. Pedigree depicting the dietary groups analyzed in the present study. Diets contained 0 mg/kg folic acid in the deficient diet, 2 mg/kg folic acid in the control diet and 6 mg/kg folic acid in the supplemented diet (*12 mice from this group were treated with ENU as a positive control for DNA damage and mutation).

As a positive control for DNA mutation, half of the male mice from the control diet ($n = 12$) at 8 weeks of age were given a single intraperitoneal injection of 75 mg/kg ethylnitrosourea (ENU, Sigma–Aldrich) dissolved in M/15 phosphate buffer, pH 6.0, and sacrificed at 18 weeks of age.

2.2. Red blood cell folate concentrations

The *Lactobacillus casei* microbiological assay was used to measure RBC folate as previously described [33]. Folate content was normalized to total protein, which was determined using the Lowry assay [33].

2.3. Cauda epididymal sperm counts

One cauda from each mouse was used for sperm collection and counting. Each cauda was placed into 1 mL of TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl and 1 mM EDTA, pH 7.4) on ice and minced with iris scissors until no obvious piece of tissue was visible by eye. The sample was then filtered through a 70 μm filter (DAKO Diagnostics Canada Inc., Mississauga, Canada) to remove any residual pieces of tissue. 10 μL of sample was diluted 1:10 in TNE buffer and counted by two independent individuals using a haemocytometer whose results were averaged to give the estimate of cauda sperm content.

2.4. Sperm chromatin structure assay

Sperm chromatin integrity was evaluated using the SCSA as previously described [34,35]. On the day of analysis, 200 μl of sperm samples with a concentration of $2.5–3 \times 10^6$ cells/mL in TNE buffer were quickly thawed at 37 °C. Immediately, sperm samples were gently mixed with 400 μl of acid-detergent solution (0.1% Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.2) on ice for 30 seconds and then stained with 1.2 mL of 6 μg/ml acridine orange staining solution in phosphate–citrate buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1 mM EDTA disodium, 0.15 mM NaCl, pH 6.0). The stained samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, Canada) immediately and data acquisition started exactly 3 min after the addition of acid-detergent solution. Duplicate aliquots were analyzed for each sample and a total of 10 000 events were collected for each measurement at a flow rate of 200–300 cells/s. A pool of normal mouse cauda sperm was used for negative and positive controls and these were analyzed every 10th sample to ensure stable cytometer parameters. Positive controls were obtained by boiling an aliquot of the pooled sperm for 3 min. Raw data were analyzed using FCS express 4 software (De Novo Software, Los Angeles, CA). Results were expressed as DNA fragmentation index (DFI). DFI is calculated as the proportion of red fluorescence to the total fluorescence for each sperm. In a sample of normal sperm, DFI values will cluster tightly while samples from animals with increased levels of sperm DNA damage will have a subpopulation of sperm with much higher DFI values. We report the proportion of cells with DFI values higher than the range of tight DFI values observed in the negative control samples (% DFI).

2.5. Isolation of DNA for mutation analysis

Sperm DNA was prepared using the methods described by Yauk et al. [36]. All DNA isolations were carried out in a laminar flow hood to minimize the risk of PCR template contamination. Briefly, sperm cells were isolated from one cauda epididymus per mouse and somatic cells were lysed with 0.15% sodium dodecyl sulfate (SDS). Sperm cells were re-suspended in 1 mL 0.2X saline–sodium citrate, 1 M 2-mercaptoethanol, 1% SDS, and digested with Proteinase K overnight at 37 °C. DNA was extracted using a standard phenol:chloroform technique followed by ethanol precipitation, and dissolved in distilled water. One μg of DNA for each mouse was digested overnight at 37 °C with the restriction enzyme *MseI* (New England Bio Labs, Ipswich, MA). All DNA dilutions used for PCR were in 5 ng/μL herring sperm DNA in 5 mM Tris–HCL, pH 8.

2.6. Single molecule PCR for detection of mutants

Template DNA was diluted to concentrations resulting in ~40–60% of the PCR amplifications producing observable products. The *Ms6-hm* allele was amplified using HM1 forward (5′-AGA GTT TCT AGT TGC TGT GA-3′) and HM1 reverse primers (5′-GAG AGT CAG TTC TAA GGC-3′) and the Roche High Fidelity PCR System, dNTPack (Roche Diagnostics, Laval, QC) on a PTC-225 DNA Engine Tetrad Thermocycler unit (MJ Research now BioRad, Mississauga, ON). PCR products were electrophoresed on 0.8% agarose gels and visualized by Southern blotting and hybridization with αP^{32} radiolabelled *Ms6-hm* probe. Wells containing observable PCR products were then electrophoresed on 40 cm long, 0.8% agarose gels at 120 V in cooled chambers for ~44–48 h. Gels were visualized by Southern blotting and hybridized with *Ms6-hm* probe. Images of the blots were normalized to achieve 10 cm spacing between the 3 and 6 kbp bands of an in-lane DNA standard (50 ng per well of 1 kbp ladder, Invitrogen). Gels were independently scored by two individuals, each blinded to the sample treatment and results observed by the other individual, by measuring the distance between each *Ms6-hm* band and the in-lane DNA standard. The average allele size per gel was calculated and bands were considered to be a mutant if they migrated either 1 mm more or less than the average allele size. Mutation frequency was calculated as the ratio of mutant bands to total bands.

2.7. Statistical analysis

Differences among the diet groups for RBC folate, cauda sperm numbers and %DFI were identified using a two-sample bootstrap test (t-Pivot method) assuming unequal variances [37,38] in R [39]. For the ESTR assay, a Poisson distribution was used to estimate the number of progenitor PCR template molecules, determined from the number of wells used and the number of positive PCR estimated for each sample. Generalized estimating equations (GEEs) [40,41] assuming a Poisson distribution for the error were used to estimate the mutation frequencies and selected pairwise comparisons using the geepack library [42–44] in R [39]. The control group for all measures was considered to be the animals fed the 2 mg/kg folate diets during early development and post-weaning.

3. Results

3.1. Red blood cell folate concentrations

Red blood cell (RBC) folate was decreased 55% in male offspring weaned to the deficient diet relative to control mice ($P < 0.01$) (Fig. 3). There were no effects of folic acid supplementation post-weaning or of folic acid deficient or supplemented diets in early development, on RBC folate. It should be noted that mice from the post-weaning diet groups were fed the control folic acid diet for three weeks prior to being killed for breeding purposes as part of another study. Treatment with ENU caused a 15% increase in RBC folate ($P = 0.05$) (Fig. 3).

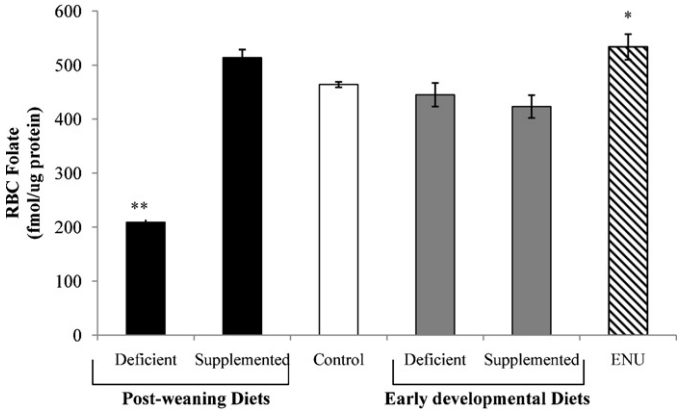

Fig. 3. Red blood cell folate in male offspring from female mice fed diets containing 0 mg/kg folic acid (deficient), 2 mg/kg folic acid (control) or 6 mg/kg folic acid (supplemented) during pregnancy/postpartum and lactation (early development). Male offspring from each dam diet group were weaned to the control diet and fed the diet for 15 weeks. A subset of male offspring from dams fed the control diet were weaned to either the deficient or supplemented diet and fed these diets for 15 weeks. Error bars represent the standard error ($^{*}P < 0.05$ for positive control group [ENU] compared to control diet group, $^{**}P < 0.01$ for weaning deficient diet group compared to control diet group).

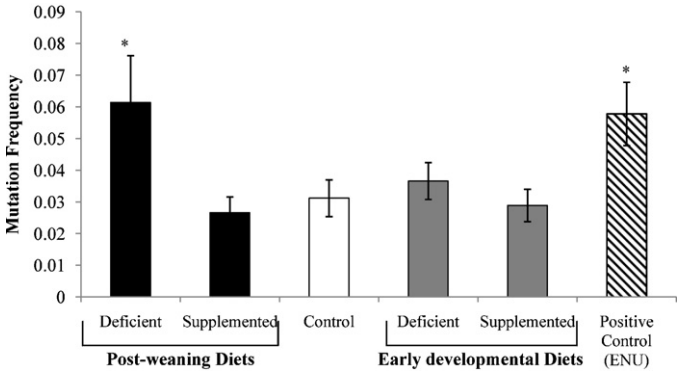

Fig. 4. Effect of folate status on tandem repeat mutation frequencies in the sperm of male offspring. Error bars represent the standard error ($^{*}P < 0.05$ compared to control diet group).

3.2. Cauda epididymal sperm counts

Male offspring weaned to the deficient diet had decreased cauda sperm number relative to control mice ($P = 0.03$) (Table 1). Exposure to the post-weaning supplemented diet did not lead to changes in cauda sperm number. Neither the early developmental deficient or supplemented diets influenced cauda sperm number (Table 1). Treatment with ENU resulted in a 42% decrease in sperm number ($P = 0.01$) (Table 1).

3.3. Sperm chromatin structure assay

The percentage of sperm with abnormal DFI (% DFI) was higher for male offspring weaned to the deficient diet compared to control mice ($P = 0.04$) (Table 1). No change in the % DFI was observed in males weaned to the supplemented diet, or in mice fed the deficient or supplemented diets in early development in comparison to control mice. Treatment with ENU resulted in a 2-fold increase in DFI $P = 0.02$).

3.4. Tandem repeat mutations

Sperm ESTR mutation frequency was increased 2-fold in male offspring weaned to the folic acid deficient diet compared to control mice ($P = 0.03$) (Fig. 4). There was no change in ESTR mutation frequency for male offspring weaned to the supplemented diet. The early developmental deficient and supplemented diets did not lead to changes in ESTR mutation frequency (Fig. 4). A 2-fold increase in ESTR mutation frequency was observed for the ENU-treated positive control group ($P = 0.01$) (Fig. 4).

4. Discussion

This study was designed to examine the effect of dietary folic acid on sperm count, chromatin structure and DNA mutation. Mice were fed folic acid deficient or supplemented diets either in early development (in utero and during lactation) or post-weaning for 15 weeks. The control diet approximates the recommended dietary allowance of folic acid for adults, which is 0.4 mg per day. The supplemented diet contained 3-fold folic acid of the control diet, an environmentally relevant amount that adults achieve through the consumption of both fortified foods and supplements [45,46].

Sperm counts are a crude measure of fertility. Decreased sperm counts generally indicate impaired spermatogenesis or increased elimination of germ cells during spermatogenesis [22,47]. Males weaned to the deficient diet had decreased cauda sperm number, similar to observed associations between folate status or intake and sperm number in men [18]. Mice lacking methylenetetrahydrofolate reductase, a key enzyme in folate metabolism, also had reduced sperm counts [48]. Folic acid deficiency could reduce sperm counts in two ways. Folic acid deficiency decreases the de novo synthesis of purines and thymidylate, which can lead to the misinsertion of dNTPs and subsequent genomic instability [49,50]. The methyl groups from folate are also required for the production of methionine, which is converted to S-adenosylmethionine, the major cellular methyl donor [51]. DNA and histone methylation may also be affected by folic acid deficiency, which could lead to changes in chromatin packaging and gene expression. Therefore DNA damage and/or altered DNA and histone methylation could lead to impaired spermatogenesis and decreased sperm counts.

The sperm chromatin structure assay is an independent predictor of successful pregnancy [52]. The assay measures the susceptibility of sperm DNA to acid-induced denaturation and produces a measure called the DNA fragmentation index [35]. An increased DFI indicates an increase in DNA strand breaks or protamine defects [52]. We found an increased percentage of sperm with abnormal DFI in male mice weaned to the deficient diet. Chromatin breaks are most likely to occur and persist during the final 3 weeks of spermatogenesis, as the cells are primarily DNA repair deficient during these latter stages. Therefore, it is of note that despite being fed a folate-replete diet for three weeks before necropsy, the deficient mice still had a significant increase in the DFI. Since folic acid deficiency leads to uracil incorporation into DNA and hence, increased DNA strand breaks, we speculate that this mechanism could be contributing to increased DFI. Direct measurement of sperm DNA uracil incorporation and associated strand breaks will clarify this issue. An increase in DFI can also indicate protamine defects [52]. During spermatogenesis, sperm DNA is condensed and the majority of histones are replaced with protamines [53]. The histones that are retained in sperm DNA are associated with the promoters of genes involved in embryo development (imprinted genes, *HOX* genes, etc.) [54]. These promoters have been found to be hypomethylated [54]. Folic acid deficiency inhibits the production of methionine and subsequently S-adenosylmethionine, the universal methyl donor, and can cause DNA hypomethylation [17]. Therefore, folic acid deficiency could affect methylation and histone replacement in sperm DNA. Our data, and data relating low folate in seminal plasma with increased

Table 1
Effects of post-weaning and early developmental dietary folic acid deficiency and supplementation on sperm count and sperm chromatin damage in male mice.

	Sperm counts		Sperm chromatin structure assay	
	# Sperm per cauda ($\times 10^6$)	*P*	% DFI	*P*
Post-weaning				
Deficient	9.3 ± 1.2	**0.03**	5.0 ± 0.9	**0.04**
Supplemented	14.0 ± 1.5	0.58	2.7 ± 0.4	0.46
Control	13.0 ± 1.1		2.6 ± 0.1	
Early development				
Deficient	11.6 ± 1.4	0.47	3.7 ± 0.5	0.32
Supplemented	10.9 ± 0.8	0.15	3.1 ± 0.7	0.27
Positive control (ENU)	7.4 ± 1.2	**0.01**	5.5 ± 1.0	**0.02**

All values are expressed as mean ± SEM. All *P* are for comparisons with control diet. Bold text indicates statistical significance.

sperm chromatin damage in men [19], illustrate that folic acid deficiency can lead to chromatin damage in sperm, which can be prevented with adequate folic acid intake.

DNA damage in sperm can lead to impaired fertility or adverse effects on early embryonic development [1]. DNA damage that becomes fixed as mutations in sperm DNA may be inherited and result in a variety of genetic disorders. Because germ cell mutations are so rare (i.e., $\sim 1.1 \times 10^{-8}$ per nucleotide per haploid genome [55]), recent efforts have focussed on quantifying mutation rates in highly variable non-coding regions of the genome [55]. Germ cell mutation frequencies can be measured at mouse ESTR loci. ESTRs are short 4–10 bp repeat units that are tandemly repeated to form long homogeneous arrays up to 20 kb in length [56,57]. They have high rates of mutation in somatic and germ cells, allowing for the determination of mutation frequencies at environmentally relevant doses using small sample sizes [58]. Although ESTR sequences are found in non-coding regions of DNA, the doubling dose for ESTR mutations is similar to that of coding DNA [59], and these loci show a dose–response relationship with both radiation and chemical mutagens [59,60]. Thus, ESTRs provide an efficient means to survey the ability of agents to cause mutations and genetic instability in the germline of mice.

In the present study we observed a 2-fold increase in ESTR mutation frequency for mice weaned to the deficient diet. We note that exposure to 75 mg/kg of the powerful germ cell mutagen ENU caused a virtually identical increase in ESTR mutation frequency. Thus, the increased mutation frequency observed by folate deficiency is substantial. ESTRs are thought to mutate through an indirect mechanism involving the formation of secondary structures that result in the gain or loss of repeat units during DNA repair or replication processes occurring in pre-meiotic stages of spermatogenesis [56,57]. Hence, the final 4 weeks prior to sample collection for ESTR analysis is not anticipated to affect the mutation outcome. The post-weaning experimental design effectively captures sperm derived from spermatogonial stem cells exposed to 12 weeks of folic acid deficiency, and a subsequent 3 weeks of deficiency for germ cells committed to spermatogenesis, including 2 weeks as pre-meiotic spermatocytes. Very recently Dubrova and co-workers [61] showed no effect of diets deficient in methyl donors on sperm ESTR mutation. However, these authors did find a non-significant 1.4-fold increase in ESTR mutation frequency [61]. In addition, their study used different diets (deficient in folic acid, choline and methionine), covering a different developmental time period, using different strains of mice and the diet was delivered over a shorter period of time [61]. Thus, these major differences likely explain the discrepancies in the two experiments.

There are three potential mechanisms that could lead to the increase in ESTR mutation frequency in the weaning folic acid deficient group. The incorporation of uracil into DNA, as a result of folic acid deficiency, is repaired by uracil glycosylase [62]. Futile cycles of uracil incorporation and repair can introduce double strand breaks, which may initiate a cell cycle checkpoint resulting in cell cycle arrest for DNA repair or programmed cell death. This can lead to global DNA polymerase pausing and subsequent formation of secondary structures at ESTR loci [56]. Folic acid deficiency also impairs de novo purine synthesis, which can lead to the misinsertion of dNTPs. Repair of these misinsertions may also initiate cell cycle arrest for repair and lead to polymerase pausing [56]. Another possibility is that folic acid deficiency may affect sperm DNA methylation, which may indirectly impact ESTR mutation frequency. For example, 5-azacytidine, a non-mutagenic agent that causes global DNA hypomethylation, causes an increase in ESTR mutation frequency in embryonic fibroblast cells in culture [57]. It has been hypothesized that chemical exposures that alter DNA methylation cause changes in chromatin conformation and affect the ability of secondary structures to be repaired at ESTR loci [57]. Since folic acid deficiency is known to cause DNA mutation, damage and global DNA hypomethylation, all of these pathways could lead to increased ESTR mutation in the germline. In our follow up studies, we will investigate the contribution of uracil incorporation and DNA methylation to ESTR mutation induction by quantifying these measures directly in the sperm of DNA of folic acid deficient mice. In addition, ESTR mutation frequencies in the descendants of these mice will be evaluated to determine if folic acid deficiency leads to transgenerational genetic instability. Studying the offspring will also provide insight into whether altered DNA methylation causes transgenerational or other effects in germ cells.

Males that were folic acid deficient during early development did not exhibit statistically significant increases in sperm chromatin damage or DNA mutation. The folic acid deficient diet was fed to female mice for four weeks prior to breeding, as well as throughout pregnancy and lactation. Male offspring were then fed the control diet from weaning for 15 weeks. Thus, these male offspring were folic acid deficient during the initial stages of germ cell development before being weaned to the control diet. Since these mice were only exposed to the folic acid deficient diet from embryo to weaning (less time than the post-weaning mice), the data suggest that early developmental exposure to deficiency has no effect or the period of exposure was not long enough to cause germ cell effects, or the effect was transient.

In contrast to the significant impacts of folate deficiency, folic acid supplementation did not affect sperm counts, sperm chromatin damage or ESTR mutation frequencies. Thus, exposure to folic acid supplementation at the level achieved in this study during early development or post-weaning is not detrimental to the male germline. We are currently measuring ESTR mutation frequencies in mice from each of the diet groups that were exposed to ENU. The results will allow us to determine whether early life or post-weaning dietary folic acid impacts chemically-induced mutation frequency in the germline.

Even though mice were fed the control diet for 3 weeks prior to sampling, RBC folate was decreased by 55% in male mice weaned to the deficient diet. Male mice weaned to the supplemented diet

did not have increased RBC folate, likely as a result of being fed the folate-replete diet. ENU treatment led to a 15% increase in RBC folate in male mice fed the control diet suggesting that ENU treatment may perturb folate metabolism. The results must be verified in future studies at which point the mechanism can be pursued.

5. Conclusions

We demonstrate that folic acid deficiency in adult male mice causes decreases in sperm numbers, and increases in germline chromatin damage and DNA mutation. Supplementation with folic acid in early development or post-weaning does not cause changes in the germ cell measures examined. Increased DNA strand breaks or hypomethylation are both potential mechanisms underlying the observed effects. Our study highlights the importance of adequate folic acid intake for male fertility and for the prevention of DNA mutation in the germline.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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