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# Dietary folic acid protects against genotoxicity in the red blood cells of mice

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ABSTRACT

Folate is an essential B vitamin required for the *de novo* synthesis of purines, thymidylate and methionine. Folate deficiency can lead to mutations and genome instability, and has been shown to exacerbate the genotoxic potential of environmental toxins. We hypothesized that a folic acid (FA) deficient diet would induce genotoxicity in mice as measured by the *Pig-a* mutant phenotype (CD24-) and micronuclei (MN) in reticulocytes (RET) and red blood cells/normochromatic erythrocytes (RBC/NCE). Male Balb/c mice were fed a FA deficient (0 mg/kg), control (2 mg/kg) or supplemented (6 mg/kg) diet from weaning for 18 wk. Mice fed the deficient diet had 70% lower liver folate ($p < 0.001$), 1.8 fold higher MN-RET ($p < 0.001$), and 1.5 fold higher MN-NCE ($p < 0.001$) than mice fed the control diet. $RET^{CD24-}$ and $RBC^{CD24-}$ frequencies were not different between mice fed the deficient and control diets. Compared to mice fed the FA supplemented diet, mice fed the deficient diet had 73% lower liver folate ($p < 0.001$), a 2.0 fold increase in MN-RET ($p < 0.001$), a 1.6 fold increase in MN-NCE ($p < 0.001$) and 3.8 fold increase in $RBC^{CD24-}$ frequency ($p = 0.011$). $RET^{CD24-}$ frequency did not differ between mice fed the deficient and supplemented diets. Our data suggest that FA adequacy protects against mutagenesis at the *Pig-a* locus and MN induction in the red blood cells of mice.



## 1. Introduction

Folate, an essential B vitamin, is required for the *de novo* synthesis of purines and thymidylate, and the remethylation of homocysteine to methionine. In conditions of folate deficiency, impaired folate metabolism can have genotoxic consequences. The rate of DNA synthesis is dependent on *de novo* purine synthesis. Reduced purine synthesis can result in cell cytostasis and cytotoxicity, as well as aberrant DNA synthesis, repair and mutagenesis [1,2]. Reduced thymidylate synthesis can result in uracil incorporation into DNA. The DNA repair machinery attempts to remove the uracil, but in the presence of a high dUTP to dTTP ratio, uracil can be incorporated again. This futile cycle of uracil incorporation and repair can ultimately lead to double DNA strand breaks and genome instability [3]. Finally, impaired folate-dependent methionine synthesis can reduce *S*-adenosylmethionine ([1]AdoMet) synthesis. AdoMet is the main cellular methyl donor and perturbations in its availability influence methylation reactions, including that of DNA, RNA and histones, which can alter gene expression and genome stability. The genotoxic consequences of folate insufficiency, either through reduced folate intake or functional folate deficiency due to polymorphisms in folate-related genes, may contribute to cancer susceptibility and other folate-dependent pathologies.

The presence of micronuclei is indicative of chromosome damage that results in partial chromosome deletion, addition or rearrangement (clastogenicity), or whole chromosome gain/loss (aneugenicity). Micronuclei represent extra-nuclear chromosome fragments or whole chromosomes that lag behind during anaphase that have not been included in the nucleus when the nuclear envelope reforms [4,5]. Higher dietary folate intake has been associated with lower micronucleus (MN) frequency in an Australian population [6]. Human lymphocytes cultured in folate deficient media exhibit DNA double strand breaks [3], reduced DNA repair [3] and

*Abbreviations:* AdoMet, *S*-adenosylmethionine; DHF, dihydrofolate; DHFR, DHF reductase; ENU, ethylnitrosourea; ESTR, Expanded Simple Tandem Repeat; FA, folic acid; MTHFR, 5,10-methylenetetrahydrofolate reductase; MN, micronucleus; CD24-, Pig-a mutant phenotype; NCE, normochromatic erythrocyte; RBC, red blood cell; RET, reticulocyte; THF, tetrahydrofolate; TYMS, thymidylate synthase.

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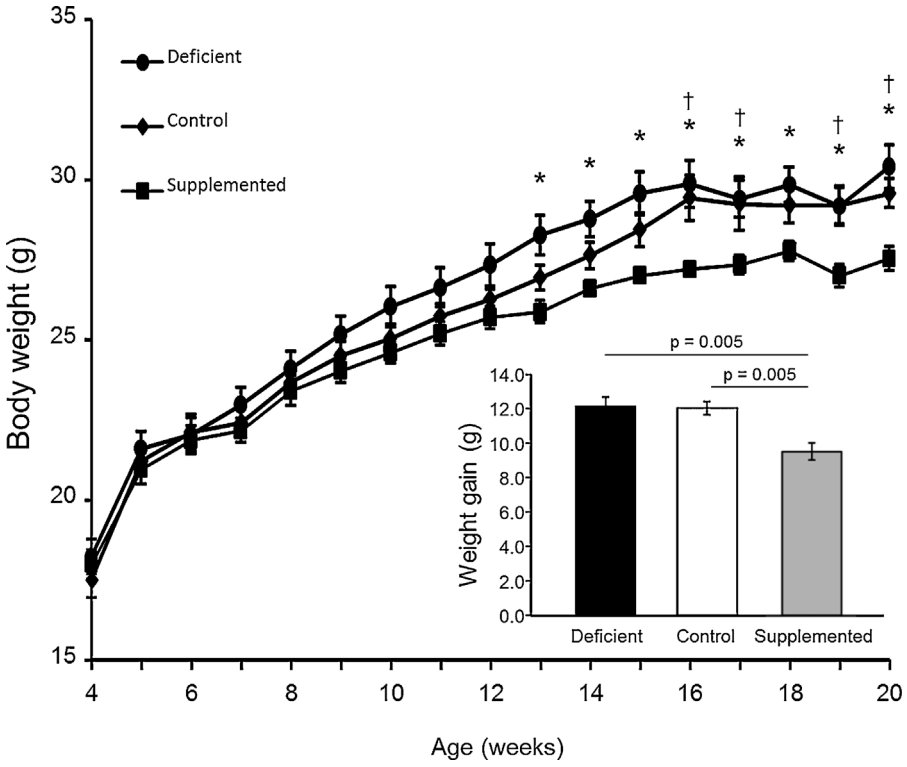


**Fig. 1.** Body weight and weight gain of male mice fed folic acid defined diets. Circles, deficient diet; diamonds, control diet; squares, supplemented diet. Body weight change over time was compared among the diet groups by Two-way ANOVA, Holm-Sidak post-hoc analysis all-ways comparisons. An asterisk (*) indicates a significant difference between the deficient and supplemented diets at that time point ($p \leq 0.002$) and a dagger (†) indicates a significant difference between the control and supplemented diets ($p \leq 0.04$). Inset, total body weight gain. Closed bars, deficient diet; open bars, control diet; grey bars, supplemented diet. Weight gain was compared among the diet groups by One-way ANOVA, Holm-Sidak post-hoc analysis for all-ways comparison; p values are indicated for comparisons that were significantly different ($p < 0.05$). Data are presented as mean ± SEM. $n = 6$/diet group.

MN formation [7–9]. Further, functional folate deficiency due to single nucleotide polymorphisms (SNPs) in folate-related genes has been associated with MN formation in lymphocytes [10].

Although the evidence for a relationship between folate and chromosome stability is strong, there is less evidence for its direct effect on gene mutations *in vivo*. Most studies have focused on the exacerbating effect of folate deficiency on mutagen-induced mutations. For example, folate deficiency was shown to exacerbate the mutagenic potential of alkylating agents at the *hprt* locus in human lymphoblastoid cell lines [11], Chinese hamster ovary cells [12] and in mice [13]. There is limited and contrasting evidence for the effect of folate deficiency alone. For example, folate deficiency was shown to cause an increased mutation frequency at the *aprt* locus in cultured Chinese hamster ovary cells, an effect that was increased further in DNA-repair deficient cells [14]. However, Balb/c mice fed a diet lacking both choline and FA for eight weeks exhibited no increase in Expanded Simple Tandem Repeat (ESTR) mutation frequencies in sperm [15]. Analysis of the *cII* mutation reporter gene in Big Blue mice fed a FA deficient diet from 3 months of age for 8 months also did not reveal significant increases in mutation frequency in colons [16]. Furthermore, mutant frequency in the Muta$^{TM}$Mouse *lacZ* reporter gene was not increased in the colons of mice whose mothers were fed a low FA versus high FA diet during pregnancy [17]. Thus, our understanding of the repercussions of folate deficiency or supplementation on gene mutations, especially *in vivo* or in a range of somatic cell types, is limited.

In an attempt to elucidate the effect of altered folate metabolism on genotoxicity, we recently showed that male Balb/c mice fed a folic acid (FA) deficient diet for 12 weeks followed by a three week wash-out period during which the mice were fed a FA sufficient diet had a higher MN frequency in normochromatic erythrocytes (MN-NCE), but not reticulocytes (RET) [18]. Balb/c mice fed a FA deficient diet for multiple generations had an amplified MN-NCE frequency and a higher MN-RET frequency than mice fed a control diet [18]. These mice also had a higher DNA fragmentation index and a 2-fold higher ESTR mutation frequency in sperm [19]. We found no difference in ESTR mutation frequency in sperm between mice fed a FA supplemented diet and a control diet, which to our knowledge, is the only study to examine the effect of FA supplementation on mutation.

Here we have used a modified study design in which mice were fed FA defined diets from weaning for 18 weeks without a control diet wash-out period to determine the direct genotoxic potential of FA deficiency on *Pig-a* mutant (CD24-) phenotype and MN frequencies in the red blood cells (RBC) of male Balb/c mice. The *Pig-a* gene is found on the X chromosome and encodes for the phosphatidylinositol glycan-class A protein that is required for glycosylphosphatidylinosital (GPI) anchored protein expression on the surface of erythrocytes. *Pig-a* mutations therefore result in GPI anchor deficiency and the frequency of RET and RBC with a *Pig-a* mutant phenotype (absence of the surface marker) can be assessed by flow cytometry using antibodies for GPI-anchored cell surface markers such as CD24 [20]. Simultaneous sampling for the *Pig-a* and MN assays allows for the determination of two genotoxic endpoints representing mutagenicity, and clastogenicity or aneugenicity, respectively, and their relationship to folate status and uracil content in DNA. The FA content of the diets used in our study was physiologically relevant: the deficient diet does not result in obvious clinical manifestations such as weight loss [18]; the control diet represents adequate dietary FA intake; and the supplemented diet approximates the combined intake of FA from fortified foods and supplements available on the Canadian market.