Effects of Altered Maternal Folic Acid, Vitamin B_{12} and Docosahexaenoic Acid on Placental Global DNA Methylation Patterns in Wistar Rats

Asmita Kulkarni, Kamini Dangat, Anvita Kale, Pratiksha Sable, Preeti Chavan-Gautam, Sadhana Joshi

Department of Nutritional Medicine, Interactive Research School for Health Affairs, Bharati Vidyapeeth University, Pune, India

Abstract

Potential adverse effects of excess maternal folic acid supplementation on a vegetarian population deficient in vitamin B_{12} are poorly understood. We have previously shown in a rat model that maternal folic acid supplementation at marginal protein levels reduces brain omega-3 fatty acid levels in the adult offspring. We have also reported that reduced docosahexaenoic acid (DHA) levels may result in diversion of methyl groups towards DNA in the one carbon metabolic pathway ultimately resulting in DNA methylation. This study was designed to examine the effect of normal and excess folic acid in the absence and presence of vitamin B_{12} deficiency on global methylation patterns in the placenta. Further, the effect of maternal omega 3 fatty acid supplementation on the above vitamin B_{12} deficient diets was also examined. Our results suggest maternal folic acid supplementation in the absence of vitamin B_{12} lowers plasma and placental DHA levels (p<0.05) and reduces global DNA methylation levels (p<0.05). When this group was supplemented with omega 3 fatty acids there was an increase in placental DHA levels and subsequently DNA methylation levels revert back to the levels of the control group. Our results suggest for the first time that DHA plays an important role in one carbon metabolism thereby influencing global DNA methylation in the placenta.

Introduction

In developing countries, micronutrient deficiencies such as folic acid and vitamin B_{12} are common and associated with poor pregnancy outcomes, having long term health effects. The peri or post-conceptional period represents a sensitive window during which suboptimal maternal micronutrients may affect feto-placental development [1]. In view of this, maternal folic acid supplementation is in operation for the last few decades in India. This is regardless of the fact that there is still a poor knowledge about potential adverse effects on a population mainly consuming a vegetarian diet that may lack vitamin B_{12} [2]. Although early results from the fortification policy indicate beneficial effects in terms of reduction in plasma homocysteine [3–5], recent reports suggest adverse effects in humans [6,7]. During growth high folic acid administration has been shown to alter dietary protein metabolism and to decrease fetal size, as compared to rats fed a control diet [8]. It has also been reported recently, that high folate intakes in vitamin B_{12} deficient mothers are shown to increase the risk of type 2 diabetes in the offspring suggesting that defects in one-carbon metabolism might be at the heart of intrauterine programming of adult disease [9].

Our earlier studies in the rat model that maternal folic acid supplementation at marginal protein levels reduces the levels of brain essential polysaturated fatty acid levels especially omega 3 fatty acids in the offspring [10,11]. Ongoing studies in our lab have also highlighted the importance of docosahexaenoic acid (an omega 3 fatty acid) during pregnancy [12,13]. Docosahexaenoic acid (DHA) is an indispensable component of all cell membranes and is incorporated in high concentrations in the membrane phospholipids of brain and retina [14] and its availability during the perinatal period is shown to be associated with long term cognitive and visual development [15,16].

It is well established that folate and vitamin B_{12} are the major determinants of one carbon metabolism in which S-adenosyl methionine (SAM) a methyl group donor is formed [17]. Dietary folate is converted in the body to 5 methyl tetrahydrofolate (5-MTHF) by the enzyme methylene tetrahydrofolate reductase (MTHFR). The transfer of methyl group from 5-MTHF to homocysteine requires vitamin B_{12} and results in the synthesis of methionine. Methionine is the precursor for SAM. Methylation of SAM is an irreversible reaction and is catalyzed by the methyltransferases. Phosphatidylcholine (PC) is critical for the delivery of important polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid from the liver to the plasma and distribution to peripheral tissues. Fig. 1 shows the interactions of folic acid, vitamin B_{12} and DHA. We have recently described that the delivery of DHA levels is low, there will be less methyl group requirement for conversion of PE-DHA to PC-DHA and may result in excess methyl group availability for other transmethylation reactions such as DNA and histone methylation.
leading to altered chromatin remodeling and gene expression [18]. We therefore hypothesize that omega 3 fatty acids play a key role in one carbon metabolism affecting global methylation levels. Placenta is an organ whose proper development and function are crucial to the health, growth, and survival of the developing fetus. A number of studies are now making important links between alterations to appropriate epigenetic regulation in the placenta and diseases of gestation and early life [19]. Examining epigenetic alterations in the placenta will prove especially important in the search for biomarkers of exposure, pathology, and disease risk and can provide critical insights into the biology of development and pathogenesis of disease [20].

The present study therefore for the first time, examines the effect of normal and excess folic acid in the absence and presence of vitamin B12 deficiency on global methylation patterns in the placenta. Further, the effect of maternal omega 3 fatty acid supplementation on the above vitamin B12 deficient diets was also examined.

Materials and Methods

This study was carried out in accordance with the CPCSEA guidelines (Committee for the purpose of control and supervision of experimental animals) Govt of India. This study was approved by the Bharati Vidyapeeth Animal Ethical Committee (IAEC/CPCSEA/258). The institute is recognized to undertake experiments on animals as per the CPCSEA, Govt of India.

Animals

Wistar albino rats (60F, 20M) of average weight 150 g were obtained from National Toxicology Center animal house. Instead of using them directly for the experimental protocol it was thought appropriate to use their progeny. They were maintained at 22°C on a controlled 12-hr light and 12 hr dark cycle with appropriate ventilation system. Animals were marked with picric acid as H (head), Back (B), Tail (T) etc for identification.

Breeding

These pups were then put for breeding at 3 months of age. Males were housed individually prior to mating to acquire cage dominance. Virgin female rats were allowed to breed (sex ratio 1:3). On the following morning the vaginal smears were taken to confirm mating. Vaginal smears were taken on a clean microscope slide using a cotton bud dipped in saline. The slides were examined under a microscope at 10× magnification. The sperm positive smear was considered a result of successful mating and considered day 0 of gestation. The pregnant dams were housed individually (in polypropylene cages of 29 x 6 x 14 cm dimensions containing rice husk as bedding material). Animals receiving Vitamin B12 deficient diets were kept in special cages to prevent coprophagy.

Out of 60 females, 47 females became pregnant and were divided randomly into 6 dietary groups. All dams were delivered by C section on day 20 of gestation.

Diets

The composition of the control and the treatment diets (Table 1) was as per AIN 93 purified diets for laboratory rodents [21]. Protein level in the control and treatment diets was 18%. Total of six isocaloric treatment diets were formulated and has been described by us recently [22,23]. Briefly four diets were formulated for examining the effects of 2 different levels of folic acid (i.e 2 and 8 mg folic acid/kg diet) during pregnancy both in the presence and absence of vitamin B12. In addition, 2 more diets were formulated to examine the effects of omega 3 fatty acid (DHA+EPA [Eicosapentaenoic acid]) supplementation on both the vitamin B12 deficient groups. Vitamin B12 deficient was obtained exclusively through
Global DNA Methylation Levels in Rat Placenta

dietary means. Vitamin free casein was used for all treatment diets. Thus there were a total of 6 groups:
Control: normal folate, normal B12, NFBD: normal folate, B12 deficient, NFBD-O: normal folate, B12 deficient, omega 3 supplemented, EFBD: excess folate, normal B12, EFBD-O: excess folate, B12 deficient, omega 3 supplemented.

The lowest level, i.e. 2 mg/kg represents the normal level of folic acid used in the control diet as per the current AIN 93 guidelines while 8 mg/kg is roughly 4 times the requirement of a normal rat. This is in accordance with the fact that folic acid requirement for Indian pregnant woman is set at 400 μg/d, which is 4 times the requirement of a non-pregnant woman. The level of omega 3 fatty acid supplementation was chosen to have an omega 6/omega 3 ratio of 1:1 which is considered to be the ideal ratio [24].

Observations recorded
During pregnancy, dam weights were recorded at day 0, 7, 14 & 20 to obtain weight gains. On day 20 of gestation the litter weight and size was recorded in each group.

Maternal plasma folic acid, vitamin B12 and homocysteine levels
Plasma vitamin B12 and plasma folic acid were measured using a radioimmunoassay kit (Diagnostic Products Corporation, USA) [25] and plasma total homocysteine was determined using the IMx System (Abbott Laboratories, IL, USA) [26].

Tissue collection and processing
Dams were dissected at day 20 of gestation and placental tissues were collected. Fetal membranes were trimmed off and the placenta was weighed. Placentas were snap frozen and stored at −80 °C until assayed.

Placental fatty acid levels
The procedure for fatty acid analysis used in our study was revised from the original method of Manku et al. that has been reported by us earlier in a separate study [27,28]. Briefly, placental tissue was homogenized with chilled PBS and centrifuged at 10000 rpm at 4 °C for 20 min. Supernatant and cell membrane fractions were separated. Transesterification of cell membrane phospholipid fraction was carried out using hydrochloric acid-methanol. These were separated using a Perkin Elmer gas chromatograph (SP 2330, 30 m capillary Supelco column. Helium was used as carrier gas at 1 mL/min. Oven temperature was held at 150 °C for 10 min, programmed to rise from 150 to 220 °C at 10°C/min, and at 220°C for 10 min. The detector temperature was 275 °C and the injector temperature was 240 °C. Retention times and peak areas were automatically computed. The column was calibrated by injecting the standard fatty acid mixture in approximately equal proportion. The data was recorded and the peaks were identified as per the retention time of the standard fatty acids (Sigma) run under the identical conditions. Fatty acids were expressed as g/100 g fatty acid. Total of 15 fatty acids were detected. Saturated fatty acids include myristic acid, palmitic acid and stearic acids, while total monounsaturated fatty acids include myristoleic, palmitoleic, oleic acid and nervonic acids. The omega 3 fatty acids included alpha linolenic acid, eicosapentaenoic acid and docosahexaenoic acid while total omega 6 fatty acids included linoleic acid, gamma linolenic acid, di-homo-gammalinolenic acid, docosapentaenoic acid and arachidonic acid.

**Table 1.** Composition of the diets.

| S.No | Diets          | Control (g/kg) | NFBD (g/kg) | EFB (g/kg) | EFBD (g/kg) | NFBD-O (g/kg) | EFBD-O (g/kg) |
|------|----------------|----------------|-------------|------------|-------------|----------------|---------------|
| 1.   | Corn Starch    | 398            | 398         | 398        | 398         | 398            | 398           |
| 2.   | Casein         | 200            | 200         | 200        | 200         | 200            | 200           |
| 3.   | Dextrinized Starch | 132         | 132         | 132        | 132         | 132            | 132           |
| 4.   | Sucrose        | 100            | 100         | 100        | 100         | 100            | 100           |
| 5.   | Soya Bean Oil | 70             | 70          | 70         | 70          | 70             | 70            |
| 6.   | Fish oil       | 0              | 0           | 0          | 0           | 45             | 45            |
| 7.   | Fiber          | 50             | 50          | 50         | 50          | 50             | 50            |
| 8.   | Mineral mixture* | 35               | 35          | 35         | 35          | 35             | 35            |
| 9.   | Vitamin mixture* | 10             | 10          | 10         | 10          | 10             | 10            |
| 10   | Folic acid     | 0.002          | 0.002       | 0.008      | 0.008       | 0.002          | 0.008         |
| 11   | Vitamin B12    | 0.025          | 0           | 0.025      | 0           | 0              | 0             |
| 12   | Cystine        | 3              | 3           | 3          | 3           | 3              | 3             |
| 13   | Choline Bitartrate | 2.5         | 2.5         | 2.5        | 2.5         | 2.5            | 2.5           |
| 14   | Tertiary Butyl Hydroquinone | 0.014 | 0.014 | 0.014 | 0.014 | 0.014 | 0.014 |
| 15   | Total Energy (kJ) | 1.57       | 1.57        | 1.57       | 1.57        | 1.57           | 1.57          |

*Mineral mixture (g/kg mixture): Calcium carbonate, 357; Potassium Phosphate, 196; Potassium Citrate, 70.78; Sodium Chloride, 78; Potassium Sulphate, 46.6; Magnesium Oxide, 24; Ferric Citrate, 6.06; Zinc Carbonate, 1.65; Manganese Carbonate, 0.63; Cupric Carbonate, 0.3; Potassium Iodate, 0.01; Sodium Selenate, 0.01; Ammonium Paramolybdate, 0.007; Sodium Metasilicate, 1.45; Chromium Potassium Sulphate, 0.275; Lithium Chloride, 0.01; Boric Acid, 0.08; Sodium Fluoride, 0.06; Nickel Carbonate, 0.03; Ammonium Vanadate, 0.006; Sucrose, 221.02.

*Vitamin mixture (g/kg mixture): Nicotinic Acid, 3; Calcium Pantothenate, 1.6; Pyridoxine-HCl, 0.7; Thiamin -HCl, 0.6; Riboflavin, 0.6; D-Biotin, 0.02; Vitamin B12 (in 0.1% Mannitol), 2.5; Vitamin E, 15; Vitamin A, 0.8; Vitamin D-3, 0.25; Vitamin K, 0.075; Folic acid, 0.2 (control) and Sucrose 974.655, was used to make total weight of the vitamin mixture to 1 kg.

Control: Normal folate, normal B12, NFBD: normal folate, B12 deficient, NFBD-O: normal folate, B12 deficient, omega 3 supplemented, EFBD: Excess folate, normal B12, EFBD-O: Excess folate, B12 deficient, EFBD-O: Excess folate, B12 deficient, omega 3 supplemented.

doi:10.1371/journal.pone.0017706.t001
Placental global methylation patterns

Genomic DNA extraction from placental tissues was carried out with the Qiagen Blood and Tissue kit. Global DNA methylation was measured using the Methy1amp™ Global DNA Methylation Quantification Kit (Epigentek Group Inc., New York, NY, U.S.A.) as we have described recently [29]. The kit yields accurate measures of methylcytosine content as a percentage of total cytosine content.

The methodology for estimation of global methylation levels used in this study takes into account methylation of all CpG’s irrespective of their position in the genome (promoter and non-promoter CpG). This is in concurrence with studies indicating that CpG methylation in intragenic and intergenic regions are also critical to gene expression [30].

Statistical Analysis

Litter means were used as the unit of analysis. Values are mean ± SD. The data were analyzed using SPSS/PC+ package (Version 11.0, Chicago IL). The treatment groups were compared with the control group by ANOVA and the post-hoc least significant difference test.

Results

Feed intake

Feed intake during pregnancy was between 15–19 g/day. There was no effect of different levels of folic acid both in the presence and absence of vitamin B₁₂ on feed intake. In contrast, feed intake in both the omega 3 fatty acid supplemented groups was lower (p<0.01) than control. Further feed intake in omega 3 fatty acid supplemented groups were also lower than those in their respective B₁₂ deficient groups (NFBD vs NFBD0, p<0.05) and (EFBD vs EFBDO, p<0.05) and has been reported by us recently [23].

Reproductive performance

There was no effect of folic acid supplementation in the presence of vitamin B₁₂ (EFBD) on weight gain of dams as compared to control during pregnancy. There was also no difference in weight gain in the dams fed omega 3 fatty acids as compared to control or any of the other treatment groups. The pup weight between groups was comparable and has been reported by us recently [23].

Maternal plasma folate, vitamin B₁₂, homocysteine and fatty acid levels

As expected, folic acid supplementation (EFB and EFBDO) increased (p<0.05) plasma folate acid as compared to controls (Table 2). Similarly animals fed a vitamin B₁₂ deficient diet had lower (p<0.05) plasma vitamin B₁₂ levels as compared to control. Homocysteine concentrations were comparable between groups (Table 2).

Placental fatty acid levels

DHA levels were significantly (p<0.05) reduced in both the NFBD and EFBDO groups as compared to control (Table 3). In contrast, supplementation with omega 3 fatty acids improved (p<0.01) DHA and omega 3 fatty acid levels but reduced arachidonic acid and omega 6 fatty acid (p<0.05) levels in NFBDO as well as EFBDO groups. MUFA (mono-unsaturated fatty acid) levels in the NFBDO group and EFBDO were reduced as compared to NFBD and EFBDO groups respectively (p<0.01 for both).

Placental global methylation levels

Global DNA methylation levels in placental tissue were reduced in the EFBD group as compared to control and NFBD group (p<0.05). In contrast, in the EFBDO group DNA methylation levels were higher (p<0.05) as compared to the EFBD and were comparable to control (Fig. 2).

Discussion

This is the first report that has examined the effect of two levels (normal and excess) of folic acid both in presence and absence of vitamin B₁₂ deficiency on global DNA methylation levels in the placenta. Our results indicate 1) altered levels of maternal micronutrients did not influence homocysteine concentrations 2) placental DHA levels were reduced in the vitamin B₁₂ deficient groups 3) excess maternal folic acid supplementation in the absence of vitamin B₁₂ results in reduced global DNA methylation levels 4) when omega 3 fatty acids were supplemented to the diet with excess maternal folic acid and vitamin B₁₂ deficiency, DNA methylation levels revert back to the levels observed in the control group.

Our findings for the first time suggest that maternal vitamin B₁₂ deficiency, both at normal and excess folic acid levels reduces placental DHA concentrations although mechanisms need to be understood. We and others have previously reported that folic acid

| Table 2. Dam plasma folate, vitamin B₁₂ and homocysteine levels. |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                | Control (n = 8) | NFBD (n = 8)   | EFB (n = 7)    | EFBDO (n = 8)  | NFBDO (n = 7)  | EFBDO (n = 6)  |
|                | Mean    | SD     | Mean    | SD     | Mean    | SD     | Mean    | SD     | Mean    | SD     |
| Folic acid (ng/ml) | 26.00  | 14.48  | 23.50  | 12.32  | 69.86   | ** 8.47 | 73.88   | ** 3.36 | 34.43   | 16.05  | 71.00   | ** 4.43 |
| Vitamin B₁₂ (pg/ml) | 287.63 | 56.33  | 192.29 | * 28.53 | 274.00  | 70.58  | 188.25  | ** 33.01 | 182.00  | ** 17.52 | 181.00  | ** 25.63 |
| Homocysteine (µmoles/L) | 7.67  | 1.24   | 7.53   | 1.42   | 6.89    | 1.48   | 7.24    | 1.35   | 8.97    | 1.34   | 7.58    | 2.21   |

*P<0.01 when compared to control;  
1P<0.05;  
2P<0.01 when compared to EFB;  
3P<0.05;  
4P<0.01 when compared to NFBD.  
AA: Arachidonic acid; Control: Normal folate, normal B₁₂; NFBD: normal folate, B₁₂ deficient, NFBD0: normal folate, B₁₂ deficient, omega 3 supplemented, EFB: Excess folate, normal B₁₂; EFBDO: Excess folate, B₁₂ deficient, EFBDO: Excess folate, B₁₂ deficient, omega 3 supplemented.

doi:10.1371/journal.pone.0017706.t002
alters DHA levels in animals [15,16]. It may be possible that vitamin B12 deficiency either results in a virtual deficiency of folate thereby affecting methyl group supply or PEMT may be epigenetically altered leading to reduced expression affecting the conversion of PE-DHA (phosphatidyl ethanolamine-DHA) to PC-DHA (phosphatidyl choline-DHA) resulting in lower DHA levels in placenta. The PC/PE ratio also modulates the activity of Delta-5 and Delta-6 desaturases involved in omega 3 and omega 6 PUFA synthesis [31].

Reports suggest that an imbalance between folate and vitamin B12 during pregnancy could influence imprinting in the embryo, perhaps by an effect on DNA methylation since folates are co-factors and co-substrates for biological methylation and nucleic acid synthesis and also function as regulatory molecules [32]. DNA methylation patterns which are largely established in-utero, induce stable changes in gene expression that may be sustained throughout the life span of an individual [33]. Associations of homocysteine with global methylation patterns are not well

| Table 3. Placental fatty acid levels in different treatment groups. |
|---------------------------------------------------------------|
|                  | Control (n = 15) | NFBD (n = 16) | EFB (n = 16) | EFBD (n = 15) | NFBDO (n = 16) | EFBDO (n = 14) |
|                  | mean  SD       | mean  SD     | mean  SD    | mean  SD     | mean  SD      | mean  SD      |
| Alpha linolenic acid | 1.86  4.72    | 0.62  0.19   | 0.40 * 0.26 | 2.13 ** 4.15 | 1.62  3.75    | 1.73  4.47    |
| Linoleic acid     | 12.77 1.39    | 12.01 1.30   | 12.19 1.73  | 13.25 ** 1.20 | 11.51 1.80    | 10.89 ** 0.97 |
| Docosahexaenoic acid | 3.73 0.88    | 2.94 * 0.99  | 3.48 0.64  | 2.76 ** 0.57 | 6.75 ** 2.17  | 7.22 ** 2.07  |
| Arachidonic acid  | 17.98 2.07    | 17.29 2.22   | 17.71 1.76  | 17.33 2.10   | 12.85 ** 3.35 | 12.49 ** 1.58 |
| Omega 3 fatty acids | 5.68 4.53    | 3.68 ** 1.09 | 4.15 0.71  | 4.98 3.95    | 9.93 ** 3.28  | 11.68 ** 3.76 |
| Omega 6 fatty acids | 31.86 3.32   | 30.49 2.90   | 31.17 2.88 | 31.60 2.85   | 27.35 ** 4.49 | 26.97 ** 1.82 |
| Monounsaturated fatty acids | 13.85 1.80 | 15.08 1.78  | 13.25 3.18  | 15.01 ** 3.05 | 12.38 ** 3.49 | 12.34 ** 2.72 |
| Saturated fatty acids | 40.61 4.82   | 43.58 2.39   | 36.80 6.51  | 41.59 ** 4.71 | 44.27 4.61    | 42.97 ** 4.40 |

*p<0.05,  **p<0.01 when compared to control;  ^p<0.05,  ^p<0.01 when compared to NFBD;  \( ^p<0.05,  \( ^p<0.01 \) when compared to EFB;  \( ^p<0.01 \) when compared to EFBD.  

Control: Normal folate, normal B12, NFBD: normal folate, B12 deficient, NFBDO: normal folate, B12 deficient, omega 3 supplemented, EFB: Excess folate, normal B12, EFBD: Excess folate, B12 deficient, EFBD: Excess folate, B12 deficient, omega 3 supplemented. 

Figure 2. Percent global DNA methylation in Wistar rat placenta.  *p<0.05 when compared to control (Normal folate, normal B12);  \( ^p<0.05 \) when compared to NFBD (Normal folate, B12 deficient);  \( \uparrow p<0.05 \) when compared to EFB (Excess folate, B12 deficient), NFBDO: normal folate, B12 deficient, omega 3 supplemented, EFB: Excess folate, normal B12; EFBD: Excess folate, B12 deficient, omega 3 supplemented.  

doi:10.1371/journal.pone.0017706.g002
established. High concentrations of homocysteine have been reported to be associated with reduced DNA methylation potential by some [34,35], while others have reported increased DNA methylation [36,37]. On the other hand, recently Bromberg et al. have reported no association between homocysteine and DNA methylation [39]. Our findings also suggest that homocysteine concentrations may not be the only determinant of global methylation levels in the placenta.

Low folic acid status is often associated with impaired DNA methylation [39], affecting gene expression in complex ways [40–43], however it is not known whether excess folic acid might have any adverse effects on these functions. In a recent study, Min et al. [44] have also shown that folic acid supplementation in vitamin B12 deficient rat did not alter hepatic SAM and SAH (S-adenosyl homocysteine) concentrations and DNA methylation. In our study, at normal folic acid there was no change in placental global DNA methylation levels. However, in contrast at excess folic acid levels in the absence of vitamin B12 it was lower as compared to control. It has been reported that effect of folate status on DNA methylation in animals and humans is tissue-, site-, and gene-specific [40,43]. Our results suggest that it may be the ratio of folic acid and vitamin B12 that may play an important role in determining global DNA methylation.

Evidence from in vivo studies has not clearly established a link between vitamin B12 and DNA methylation. However it has been demonstrated in the animal model that a B12 deficient diet, disturbs normal homeostasis of one-carbon metabolism in the colonic mucosa and results in diminished genomic DNA methylation and increased uracil misincorporation in DNA [45]. Further, it has recently been shown that gene expression patterns change under B12 deficient conditions and are recovered by dietary methionine supplementation to B12 deficient rats [46].

For the first time this study has shown that supplementation with omega 3 fatty acids in excess folic acid and vitamin B12 deficient group increased placental global DNA methylation to control levels. During early development, there are two waves of demethylation which are followed by a gradual increase in de novo methylation in the embryonic and extraembryonic (which includes the placenta) tissues [47]. Deficiencies of vitamin B12 or other abnormalities within the one carbon cycle have been implicated in the development of such placental diseases [48]. Our findings suggest that placental maturation and development may be hampered due to vitamin B12 deficiency and absence of DHA leading to lower total global methylation (hypo methylation). DHA is reported to play an important role in development and maturation of vital tissues such as brain and placenta [49,50]. Our findings indicate that DHA supplementation restores (increases) the global methylation levels to control levels suggesting that that omega 3 fatty acids especially DHA plays an important role in determining methylation levels in the placenta. Further, our results are in line with previous studies in the rat model which have demonstrated that supplementation with omega-3 fatty acids during pregnancy [51] or postnatal life [52] could prevent or limit adverse outcomes of fetal programming. Further studies on gene-specific methylation involved in placental growth and pathology will throw light on the mechanisms to explain the current data.

The methodology for estimation of global methylation levels used in this study takes into account methylation of all CpG’s irrespective of their position in the genome (promoter and non-promoter CpG). This is in concurrence with studies indicating that CpG methylation in intragenic and intergenic regions are also critical to gene expression [30]. Although this study has not examined methylation at gene specific level, our lab has initiated studies to examine the epigenetic changes occurring at genes associated with one carbon metabolism. Understanding these mechanisms may help in elucidating pathways associated with adverse pregnancy outcomes. In conclusion, changes in maternal micronutrients such as folate, vitamin B12 and omega-3 fatty acids could alter the availability of these key metabolites of one carbon cycle in the fetus, providing a direct link between maternal nutrition and placental gene methylation.

Acknowledgments

We acknowledge the help of Mr. Vinayak Dhavale, who took care of the animals. We also thank Dr (Mrs) Vijaya Pandit for her suggestions in carrying out the animal protocol.

Author Contributions

Conceived and designed the experiments: PCG A. Kale SJ. Performed the experiments: A. Kulkarni KD PS A. Kale. Analyzed the data: PCG A. Kale SJ. Contributed reagents/materials/analysis tools: A. Kulkarni KD PS A. Kale. Wrote the paper: PCG SJ.

References

1. Owens S, Fall CH (2008) Consequences of poor maternal micronutrition before and during early pregnancy. Trans R Soc Trop Med Hyg 102: 103–104.
2. Yajnik CS, Deshpande SS, Jackson AA, Redburn H, Rao S, et al. (2008) Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia 51: 29–38.
3. Choumenkovitch SF, Jacques PF, Nadeau MR, Wilson PW, Rosenberg IH, et al. (2001) Folic acid intake from fortification in United States exceeds predictions. J Nutr 132: 2792–2796.
4. Hertrampe E, Cortes F, Ericsson JD, Cayazzo M, Freire W, et al. (2003) Consumption of folic acid fortified bread improves folic status in women of reproductive age in Chile. J Nutr 153: 3166–3169.
5. Jacques PF, Selhub J, Bosron W, Wilson P, Rosenberg I (1999) The effects of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 340: 1449–1454.
6. Windham GC, Shaw GM, Todoroff K, Swan SH (2000) Mecarriage and use of multivitamins or folic acid. Am J Med Genet 90: 261–262.
7. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM, et al. (2000) Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. Obstet Gynecol 59: 519–524.
8. Achiou M, Varela-Moreiras G, Alonso-Aperte E (2000) High dietary folic acid supplementation in weanling rats affects dietary protein metabolism: implications in the methionine cycle; in Mato JM, Caballero A (eds) Workshop on Methionine Metabolism: Molecular and Clinical Implications. Master Line. pp 303–317.
9. Yajnik CS, Deshmukh US (2008) Maternal nutrition, intra uterine programming and consequential risks in the offspring. Rev Endocr Metab Disord 9: 203–211.
10. Rao S, Joshi S, Kale A, Henge M, Mahakad S (2006) Maternal folic acid supplementation to dams on marginal protein level alters brain fatty acid levels of their adult offspring. Metabolism 55: 628–634.
11. Pita ML, Delgado MJ (2000) Folate administration increases n-3 polyunsaturated fatty acids in rat plasma and tissue lipids. Thromb Haemost 84: 420–425.
12. Kikari AS, Mehedendie SS, Daung KD, Yadav HR, Kulakarni AV, et al. (2009) Long chain polyunsaturated fatty acids in mothers and term babies. J Perinat Med 37: 513–518.
13. Daung KD, Mehedendie SS, Yadav HR, Kikari AS, Kulakarni AV, et al. (2009) Long-Chain Polyunsaturated Fatty Acid Composition of Breast Milk in Pre-Eclamptic Mothers. Neonatology 97: 190–194.
14. Fiesler SJ, Anderson RE (1983) Chemistry and metabolism of lipids in the vertebrate retina. Prog Lipid Res 22: 79–131.
15. Holland IR, Smith L, Saarem K, Saugstad OD, Drevon CA (2003) Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children’s IQ at 4 years of age. Pediatrics 111: e39–44.
16. Whalley LJ, Fox HC, Wahle KW, Starr JM, Deary IJ (2004) Cognitive aging, childhood intelligence, and the use of food supplements: possible involvement of n-3 fatty acids. Am J Clin Nutr 80: 1650–1657.
17. Selhub J (1999) Homocysteine metabolism. Annu Rev Nutr 19: 217–46.
18. Kale A, Naphade N, Sapkal S, Kamaraju M, Pillai A, et al. (2010) Reduced folic acid, vitamin B12 and docosahexaenoic acid and increased homocysteine levels in rat placenta tissues [47]. Deficiencies of vitamin B12 or other abnormalities within the one carbon cycle have been implicated in the development of such placental diseases [48]. Our findings suggest that placental maturation and development may be...
and cortisol in never-medicated schizophrenia patients: Implications for altered one-carbon metabolism. Psychiatry Res 175: 47–53.
19. Kim JM, Hong K, Lee JH, Lee S, Chang N (2009) Effect of folate deficiency on placental DNA methylation in hyperhomocysteinemic rats. J Nutr Biochem 20: 172–176.
20. Maccani MA, Marsit CJ (2009) Epigenetics in the Placenta. Am J Reprod Immunol 62: 78–89.
21. Reyes PG, Niehen FH, Fahey GC (1993) AIN-93 purified diets for laboratory rodents: Final report of the American Institute of nutrition, Ad-hoc writing committee on the reformulation of the AIN-76 rodent diet. J Nutr 123: 1939.
22. Dangat K, Kale A, Joshi S (2011) Maternal supplementation of omega 3 fatty acids to micronutrient imbalanced diet improves lactation in rat. Metabolism: Clinical and Experimental. In press.
23. Roy S, Kale A, Dangat K, Sahle P, Kulkarni A, Joshi S (2011) Maternal micronutrients (folic acid and vitamin B12) and omega 3 fatty acids: Implications for neurodevelopmental risk in the rat offspring. Brain Dev. In press.
24. Smopoulos AP (1999) Essential fatty acids in health and chronic disease. Am J Clin Nutr 70: 560S–5698.
25. Lee DSC, Griffiths BW (1985) Serum vitamin B12 assay methods: A review. Clin Biochem 18: 261–266.
26. Zighetti ML, Chantarangkul V, Tripodi A, Mannucci PM, Catteaneo M (2002) Determination of total homocysteine in plasma: comparison of the Abbott IMx immunoassay with high Performance liquid chromatography. Haematologica. 87: 89–94.
27. Manku MS, Horrobin DF, Huang YS, Morse N (1983) Fatty acids in plasma and red cell membranes in normal humans. Lipids 18: 906–908.
28. Kulkarni A, Mehandale S, Yadav H, Joshi S (2011) Reduced placental docosahexaenoic acid levels associated with increased levels of sFlt-1 in preclampsia. PLEFA 84: 51–55.
29. Kulkarni A, Chavan-Gautam P, Mehandale S, Yadav H, Joshi S (2010) Global DNA methylation patterns in placenta and its association with maternal hypertension in pre-eclampsia. DNA Cell Biol, In press.
30. Fazzari MJ, Greally JM (2004) Epigenomics: beyond CpG islands. Nat Rev Genet 5: 449–458.
31. Smith DA, Kim VI, Refum H (2008) Is folic acid good for everyone? Am J Clin Nutr 87: 517–533.
32. Selley ML (2007) A metabolic link between S-adenosylhomocysteine and polyunsaturated fatty acid metabolism in Alzheimer’s disease. Neurobiol Aging 28: 1834–1839.
33. Waterland RA, Jirtle RL (2004) Early nutrition, epigenetic changes at one-carbon metabolism and the role of fatty acid-binding proteins. J Nutr Biochem 8: 517–533.
34. Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, et al. (2000) Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 275: 29318–29323.
35. Castro R, Rivera I, Struyf EA, Jansen EEW, Ravasco P, et al. (2003) Increased Homocysteine and S-Adenosylhomocysteine Concentrations and DNA Hypomethylation in Vascular Disease. Clin Chem 49: 1292–1296.
36. Bönisch D, Leuz B, Reulbach U, Kornhuber J, Bleich S et al (2004) Homocysteine associated genomic DNA hypomethylation in patients with chronic alcoholism. J Neural Transm 111: 1611–1616.
37. Sharma P, Kumar J, Garg G, Kumar A, Patowary A, et al. (2008) Detection of Altered Global DNA Methylation in Coronary Artery Disease Patients. DNA Cell Biol 27: 357–365.
38. Bromberg A, Levine J, Nemetz B, Belmaker RH, Agam G (2008) No association between global leukocyte DNA methylation and homocysteine levels in schizophrenia patients. Schizophr Res 101: 56–57.
39. Friis S, Choi SW (2002) Gene-nutrient interactions and DNA methylation. J Nutr 132: 2382S–2387S.
40. Kim YI (2005) Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. J Nutr 135: 2703–2709.
41. Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33: 245–254.
42. Juhaser VS, Wagner C, Terpel JK (2001) Impact of extracellular folate levels on global gene expression. Mol Pharmaco 60: 1208–1295.
43. Mccabe DC, Caullil MA (2000) DNA methylation, genomic silencing, and links to nutrition and cancer. Nutr Rev 63: 183–185.
44. Min H (2009) Effects of dietary supplementation of high-dose folic acid on biomarkers of methylating reaction in vitamin B12-deficient rats. Nutr Res Pract 3: 122–127.
45. Friis S, Choi SW (2005) The potential cocarcinogenic effect of vitamin B12 deficiency. Clin Chem Lab Med 43: 1108–1113.
46. Urkawa A, Katsushika K, Ogata A, Kawazu T, Maeda N, et al. (2009) Change of epigenetic control of cystathionine beta-synthase gene expression through dietary vitamin B12 is not recovered by methionine supplementation. J Nutrigenet. Nutrigenomics 2: 29–36.
47. Geiman TM, Muegge K (2010) DNA Methylation in Early Development. Mol Reprod Dev 77: 105–113.
48. Ray JG, Laskin CA (1999) Folic acid and homocyst(e)ine metabolic defects and the risk of placental abruption, pre-eclampsia and spontaneous pregnancy loss: A systematic review. Placenta 20: 519–529.
49. de Urrquia AM, Liu S, Sjöberg M, Zetterström RH, Griffiths W, et al. (2000) Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. Science 290: 2140–2144.
50. Dutta-Roy AK (1997) Fatty acid transport and metabolism in the feto-placental unit and the role of fatty acid-binding proteins. J Nutr Biochem 8: 548–557.
51. Grenier E, Ziv E, Delvin E, Leduc L, Spahis S, et al. (2008) N3-fatty acids on one-carbon metabolism and cortisol in never-medicated schizophrenia patients: Implications for altered one-carbon metabolism. Psychiatry Res 175: 47–53.
52. Kim YI (2005) Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. J Nutr 135: 2703–2709.
53. Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33: 245–254.
54. Juhaeri VS, Wagner C, Terpel JK (2001) Impact of extracellular folate levels on global gene expression. Mol Pharmaco 60: 1208–1295.
55. Mccabe DC, Caullil MA (2000) DNA methylation, genomic silencing, and links to nutrition and cancer. Nutr Rev 63: 183–185.
56. Min H (2009) Effects of dietary supplementation of high-dose folic acid on biomarkers of methylating reaction in vitamin B12-deficient rats. Nutr Res Pract 3: 122–127.
57. Friis S, Choi SW (2005) The potential cocarcinogenic effect of vitamin B12 deficiency. Clin Chem Lab Med 43: 1108–1113.
58. Urkawa A, Katsushika K, Ogata A, Kawazu T, Maeda N, et al. (2009) Change of epigenetic control of cystathionine beta-synthase gene expression through dietary vitamin B12 is not recovered by methionine supplementation. J Nutrigenet. Nutrigenomics 2: 29–36.
59. Geiman TM, Muegge K (2010) DNA Methylation in Early Development. Mol Reprod Dev 77: 105–113.
60. Ray JG, Laskin CA (1999) Folic acid and homocyst(e)ine metabolic defects and the risk of placental abruption, pre-eclampsia and spontaneous pregnancy loss: A systematic review. Placenta 20: 519–529.