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Effect of folate deficiency on placental DNA methylation in hyperhomocysteinemic rats

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Abstract

We report that the maternal folate status can influence folate-mediated one-carbon metabolism and DNA methylation in the placenta. Thirty-six female Sprague–Dawley rats were divided into the following three dietary groups: folate-supplemented (FS; 8 mg/kg folic acid, n=12), homocystine- and folate-supplemented (HFS; 0.3% homocystine and 8 mg/kg folic acid, n=12) and homocystine-supplemented and folate-deficient (HFD; 0.3% homocystine and no folic acid, n=12). The animals were fed their experimental diets from 4 weeks prior to mating until Day 20 of pregnancy (n=7–9 per group). The HFS diet increased the plasma homocysteine and placental DNA methylation but did not affect plasma folate, vitamin B-12, *S*-adenosyl methionine (SAM) or *S*-adenosyl homocysteine (SAH) levels, or the SAM/SAH ratio in the liver and placenta compared with the FS diet. The HFD diet induced severely low plasma folate concentrations, with plasma homocysteine levels increasing up to 100 μmol/L, and increased hepatic SAH and decreased placental SAM levels and SAM/SAH ratio in both tissues, with a concomitant decrease in placental DNA methylation. Placental DNA methylation was significantly correlated with placental (γ=0.819), hepatic (γ=0.7) and plasma (γ=0.752) folate levels; plasma homocysteine level (γ=–0.688); hepatic SAH level (γ=–0.662) and hepatic SAM/SAH ratio (γ=0.494). These results suggest that the maternal folate status in hyperhomocysteinemic rats influences the homeostasis of folate-mediated one-carbon metabolism and the methyl pool, which would, in turn, affect placental DNA methylation by altering the methylation potential of the liver.

Keywords: DNA methylation; Folate; Homocysteine; SAM/SAH; Placenta; Pregnancy

1. Introduction

Pregnancy is associated with increases in cellular proliferation and one-carbon metabolism resulting from uterine enlargement, expansion of blood volume, placental development and fetal growth [1,2]. Folate plays a major coenzymatic role in one-carbon metabolism and, thus, in the synthesis of nucleotides and amino acids, and also in DNA methylation, all of which are important to the increased needs during pregnancy for cellular proliferation and one-carbon metabolism [3]. Abnormally low circulating levels of folate can cause an accumulation of homocysteine with a concomitant reduction in *S*-adenosyl methionine (SAM), which leads to an impaired and possibly insufficient capacity for DNA methylation [4]. The mechanisms underlying placental vasculopathy may include low concentrations of folic acid and vitamin B-12 [5]. Also, hyperhomocysteinemia may be associated with an increased risk of deep venous thrombosis [6], preeclampsia [7], spontaneous pregnancy loss [8], intrauterine death [9], placental disease [10] and neural tube defects [11].

DNA methylation is one of several epigenetic mechanisms that regulate genomic programming and imprinting during embryogenesis [12]. It was recently suggested that an altered intrauterine milieu induced by an abnormal maternal environment (e.g., uteroplacental insufficiency, maternal

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malnutrition, smoking and diabetes) affects hepatic one-carbon metabolism and subsequent DNA methylation, which thereby alters chromatin dynamics and leads to persistent changes in gene expression [13]. Furthermore, the importance of methyl donors, including folate, in DNA methylation and embryonic development has been demonstrated [14].

While numerous studies have addressed the correlations between pregnancy, folate nutrition and metabolism, the effect of folate on DNA methylation patterns in the placenta is poorly characterized.

The abovementioned observations prompted us to investigate the effects of maternal folate status on placental DNA methylation and folate-mediated one-carbon metabolism in the plasma, liver and placenta of hyperhomocysteinemic rats. We also characterized the associations between placental DNA methylation and folate metabolites.

2. Materials and methods

2.1. Animal preparation

The animal experiments followed a protocol approved by the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). Thirty-six animals were divided into the following three dietary groups: folate-supplemented (FS; 8 mg/kg folic acid, n=12), homocystine- and folate-supplemented (HFS; 3 g/kg homocystine and 8 mg/kg folic acid, n=12) and homocystine-supplemented and folate-deficient (HFD; 3 g/kg homocystine and no folic acid, n=12). The diets used are detailed elsewhere [15]. Female Sprague–Dawley rats (Orient Company, Seoul, Korea) were fed the experimental diets beginning at 5 weeks of age. Four weeks later, the rats were mated with males of the same strain. Mating was confirmed by detection of a vaginal plug, and this was denoted as day 0. The female rats continued to receive the same diets until pregnancy Day 20 (n=7–9 per group), at which time they were anesthetized with diethyl ether; blood was collected in EDTA-containing tubes and centrifuged and the liver and placenta were dissected, weighed and stored at −70°C until analysis. Pregnancy rates and placental numbers were counted.

2.2. Analysis of plasma homocysteine, vitamin B-12 and folate and tissue folate

Blood samples were drawn from a heart puncture and promptly centrifuged at 1750g for 15 min at 4°C. Plasma homocysteine was analyzed by high-performance liquid chromatography (HPLC) (Waters 474, Milford, MA, USA), with fluorescence detection according to the method developed by Araki and Sako [16]. Plasma vitamin B-12 and folic acid were measured with the ^{57}Co vitamin B-12 and ^{125}I folic acid Dualcount solid-phase-no-boil radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA).

The folate levels in the liver and placental tissues were measured by a microbiological assay using *Lactobacillus casei* (ATCC 7469) after conjugase treatment as reported by Hyun et al. [17] with slight modifications. Glycerol stock suspensions of the *L. casei* culture were diluted in 0.9% sterile saline and added to growth media. Potassium phosphate-ascorbate buffer (0.1 M) and the assay media were added to a standard 96-well microplate, with experimental samples added at serial dilutions. The microplate was incubated overnight in a 37°C incubator and then read on the following day using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA).

2.3. Analysis of SAM and S-adenosyl homocysteine in the liver and placenta

The levels of SAM and *S*-adenosyl homocysteine (SAH) in the tissues were analyzed by HPLC (Waters 474) using a slightly modified version of the ultraviolet detection method developed by Wang et al. [18].

2.4. DNA methylation measurement by pyrosequencing

Genomic DNA (gDNA) was isolated from the rat placenta using the PUREGENE Cell and Tissue kit (Gentra, Minneapolis, MN, USA) as we described previously [19]. The bisulfite modification of gDNA was performed using the CpGenome DNA modification kit (CHEMICON, Temecula, CA, USA), after which the ID element was amplified by polymerase chain reaction in a 25-μl volume containing AmpliTaq Gold with 30 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min using the forward primer 5′-GGGTTGGGGATTTAG-3′ at 0.1 μM and the biotinylated reverse primer 5′-AACCCAAAACCTTA-3′. The sequencing reaction was performed automatically (PSQ 96MA system, Pyrosequencing, Uppsala, Sweden) using the pyrosequencing primer 5′-GGGGATTTAGTTTAGTGGT-3′.

2.5. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (Microsoft Windows version 11.0). Statistical differences were determined by Student's t test or Fisher's Exact test. The Pearson correlation coefficient (γ) was used to evaluate the relationships between DNA methylation and various folate metabolites. Data are presented as mean and S.E.M. values, and significance was accepted at a probability of P<.05.

3. Results

3.1. Weight gain, food intake, organ weight, pregnancy outcome and plasma concentrations of folate, vitamin B-12 and homocysteine

The food intake, weight gain, food efficiency ratio and weights of livers in the FS and HFD group did not differ from those in the HFS group (data not shown). Table 1 lists the pregnancy outcomes in experimental rats fed the three experimental diets. Pregnancy rates and placental numbers

Table 1
Pregnancy outcomes in experimental rats fed three experimental diets [a]

Group	FS	HFS	HFD
No. of mated females	12	12	12
Pregnancy at term [b]	75.0%	58.3%	66.7%
No. of placenta per mother [c]	12.2±0.94	11.0±0.89	11.9±1.00
Placental weights (g)	0.38±0.01	0.43±0.04	0.39±0.02

[a] FS, 8 mg/kg folic acid; HFS, 0.3% homocystine and 8 mg/kg folic acid; HFD, 0.3% homocystine and no folic acid.
[b] Pregnancy rates did not differ significantly from those in the HFS group according to Fisher's exact test.
[c] Placental numbers and weights (mean±S.E.M. values) did not differ significantly from those in the HFS group according to Student's *t* test.

and weights in the FS and HFD group did not differ from those in the HFS group. Plasma homocysteine levels were significantly higher (298%) in HFS group than in FS group (Table 2), and plasma folate was significantly lower (8%) and homocysteine levels were significantly higher (433%) in HFD group than in HFS group. However, there were no differences in plasma folate between FS and HFS. Vitamin B-12 in the FS and HFD group did not differ from those in the HFS group.

3.2. Folate-mediated one-carbon metabolism

Folate-derived methyl groups contribute directly to SAM synthesis. We examined the levels of folate, SAM and SAH, and the SAM/SAH ratio in the liver (Table 3) and placenta (Table 4) of the rats. The folate level (76%) was lower in HFS livers than in FS livers, and the folate level (17%) and SAM/SAH ratio (38%) were lower and the SAH level (169%) was higher in HFD livers than in HFS livers. However, there were no differences in the SAH level and SAM/SAH ratio between FS and HFS, and in SAM level between all groups.

The levels of folate, SAM and SAH, and the SAM/SAH ratio in the placenta did not differ between FS and HFS. The levels of folate (35%) and SAM (68%), and the SAM/SAH ratio (49%) in the placenta were lower in the HFD group than in the HFS group. The placental SAH level did not differ significantly between HFS and HFD.

3.3. Placental DNA methylation

We found that gDNA methylation in the placenta was significantly higher in HFS group than in FS group (Fig. 1, *P*=.001). In contrast, placental DNA methylation was significantly lower in the HFD group than in the HFS group (*P*=.001).

Table 2
Folate, vitamin B-12 and homocysteine concentrations in pregnant rats fed three experimental diets

Group	FS (n=9)	HFS (n=7)	HFD (n=8)
Folate (nmol/L)	34.29±2.31 [a]	25.81±3.43	2.10±0.41 **
Vitamin B-12 (pmol/L)	2.50±0.22	3.23±0.31	2.83±0.09
Homocysteine (μmol/L)	7.61±0.49 *,[b]	22.69±3.29	98.33±6.49 *

[a] Data are mean±S.E.M. values.
[b] Values differed significantly from those in the HFS group.
* *P*<.01.
** *P*<.001.

Table 3
Hepatic folate, SAM and SAH concentrations, and SAM/SAH ratio in pregnant rats fed three experimental diets

Group	FS (*n*=9)	HFS (*n*=7)	HFD (*n*=8)
Folate (nmol/g tissue)	12.90±0.72[a,b, **]	9.86±0.44	1.70±0.21 ***
SAM (μmol/g tissue)	13.60±1.90	8.69±1.34	6.06±1.15
SAH (μmol/g tissue)	8.18±1.20	6.43±0.56	10.89±1.35 *
SAM/SAH ratio	1.78±0.25	1.44±0.26	0.55±0.08 **

[a] Data are mean±S.E.M. values.
[b] Values differed significantly from those in the HFS group.
* *P*<.05.
** *P*<.01.
*** *P*<.001.

3.4. Correlations

Table 5 lists the correlation coefficients between placental DNA methylation and other parameters. Placental DNA methylation was significantly correlated with folate levels in the placenta (*r*=0.819, *P*<.0001), plasma (*r*=0.752, *P*=.0003) and liver (*r*=0.7, *P*=.0012), and with the plasma homocysteine level (*r*=−0.688, *P*=.0016). We also found that placental DNA methylation was associated with the concentration of hepatic SAH (*r*=−0.662, *P*=.0028) and the hepatic SAM/SAH ratio (*r*=0.494, *P*=.0374).

4. Discussion

In this study, we found that maternal folate deficiency affected the intra- and extracellular folate-mediated one-carbon metabolism and reduced placental DNA methylation in hyperhomocysteinemic rats. To the best of our knowledge, this is the first study which reports that placental DNA methylation is strongly related to the plasma and tissue folate levels and to the hepatic levels of methylation intermediates.

We found that dietary homocystine supplementation in a folate-supplemented (HFS) status enhanced plasma homocysteine level, but caused no changes in the folate or vitamin B-12 levels. The HFD diet used in the present study induced

Table 4
Placental folate, SAM, and SAH concentrations, and SAM/SAH ratio in pregnant rats fed three experimental diets

Group	FS (*n*=9)	HFS (*n*=7)	HFD (*n*=8)
Folate (nmol/g tissue)	1.33±0.08 [a]	1.56±0.12	0.54±0.08 [b, **]
SAM (μmol/g tissue)	3.29±0.32	2.99±0.27	2.03±0.15 *
SAH (μmol/g tissue)	1.78±0.13	1.65±0.26	2.08±0.15
SAM/SAH ratio	1.94±0.26	2.14±0.46	1.04±0.14

[a] Data are mean±S.E.M. values.
[b] Values differed significantly from those in the HFS group.
* *P*<.05.
** *P*<.001.

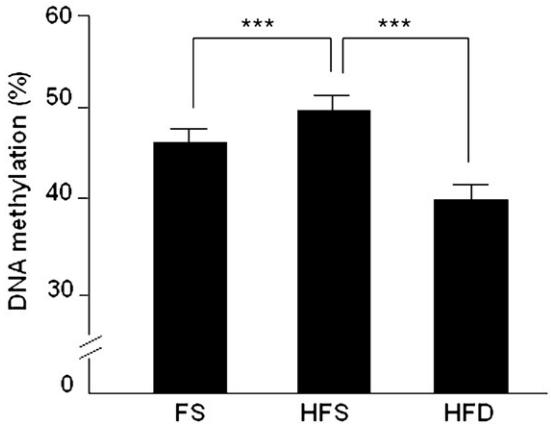

Fig. 1. Global DNA methylation in the placenta of pregnant rats fed three experimental diets. Data are mean and SEM values. ***P<.001, relative to those in the HFS group.

not only severely lowered plasma folate concentrations but also hyperhomocysteinemia of up to 100 μmol/L in pregnant rats. The folate levels were lower in HFD group than in HFS group in both the liver and placenta. These results are in good agreement with our previous report [15] that dietary homocystine supplementation caused hyperhomocysteinemia and that folate supplementation in a homocystine-supplemented status caused an increase in hepatic SAM/SAH ratio in adult male rats.

DNA methylation is known to be altered by several factors, including changes in the concentrations of SAM and SAH [20,21]. It has also been reported that folate deficiency enhances plasma homocysteine concentrations and reduces SAM/SAH ratios in the liver [22]. In the present study, however, we found that SAM/SAH ratios were reduced not only in the liver but also in the placenta in rats of HFD group compared with HFS group. Hepatic SAH was higher, and placental SAM was lower in HFD group than in HFS group with a concomitant decrease in the placental DNA methylation. This is consistent with the report by Sibani et al. [20] that a diet deficient in folate and choline reduced the SAM level and DNA methylation in the small intestine of C57Bl/6JApc$^{Min/+}$ mice. Caudill et al. [23] also reported that elevated SAH levels lead to decreased DNA methylation in the liver, kidney, brain and testes of cystathionine-β-synthase-deficient mice fed methyl-deficient diets, irrespective of whether tissue SAM levels were unchanged or decreased. These findings indicate that despite the tissue-specific SAM and SAH responses to the changes in the folate status, the decreased level of placental transmethylation activity (as indicated by the low SAM/SAH ratio) in our study, may be attributed to the folate deficiency. This led us to speculate that the maternal folate status can induce changes in folate-mediated one-carbon metabolism and in transmethylation activity leading to alterations in placental DNA methylation.

Interestingly enough, in another result of our study, we found that despite that homocystine supplementation in the folate-supplemented status did not affect the SAM, SAH and the SAM/SAH ratios in the liver and placenta, placental DNA methylation was unexpectedly higher in the HFS group than that in the FS group. We speculated that this is a result of a compensatory mechanism in which folate and homocystine in the diet modifies the process of DNA methylation during pregnancy. In our pregnant rats, a sufficiency in methyl donors with a supplemented homocystine in the diet may have increased the potential for DNA methylation to provide normal epigenetic programming, cell differentiation and development. The precise mechanisms and consequences of such hypermethylation are currently unknown and need to be investigated in a further study.

In regard to the effect of folate status on placental health and pregnancy outcome, many studies have been reported [10,24,25]. Steegers-Theunissen et al. [26], for example, reported that apoptosis is higher in placental cells cultured in folic-acid-deficient medium than in those cultured in folic-acid-sufficient medium. However, few studies have been done in regard to the relations between DNA methylation and pregnancy outcomes. Fetal development and growth are closely connected with the placental folate metabolism including DNA methylation [13,27,28]. It has been reported that a decrease of DNA methylation caused abnormal development and embryo death in embryonic stem cell [29] and incomplete closure of the cephalic neural tube in rat embryos in vitro [30]. In our study, despite decrease of DNA methylation caused by folate deficiency, there were no changes in the pregnancy rates and placental numbers and weights. We do not yet know at this stage whether folate deficiency has any effects on fetal growth and development. Further studies are needed to investigate this effect.

We additionally found that placental DNA methylation was strongly related to the folate levels in the placenta (r=0.819), liver (r=0.7) and plasma (r=0.752), and plasma homocysteine levels (r=−0.688). Placental DNA methyla-

Table 5
Pearson's correlation coefficients between DNA methylation of the placenta and other parameters in experimental rats

	DNA methylation	
	r	P
Folate		
Plasma	0.752	.0003
Liver	0.700	.0012
Placenta	0.819	<.0001
SAM		
Liver	0.310	.2104
Placenta	−0.008	.976
SAH		
Liver	−0.662	.0028
Placenta	0.363	.1387
SAM/SAH ratio		
Liver	0.494	.0374
Placenta	−0.316	.202
Plasma homocysteine	−0.688	.0016
Plasma vitamin B12	0.243	.3321

tion was highly correlated with the SAH level ($r=-0.662$) and SAM/SAH ratio ($r=0.494$) in the liver. Although the applied diets had no significant effects on the SAM level in the liver, they were found to affect the SAM/SAH ratio (which is an indicator of the cellular methylation capacity), consequently leading to an altered DNA methylation. To our surprise, we found no correlation between DNA methylation and methionine intermediates in the placenta. Given that folate deficiency affected placental SAH levels and SAM/SAH ratios, the present data do not rule out the possibility that DNA methylation and methionine intermediates in the placenta are strongly correlated.

Together, our results strongly indicate that dietary folate intake in rats affects the folate-mediated one-carbon metabolism and the methyl pool, which, in turn, affects the placental DNA methylation. This study warrants further investigation on the issues regarding the consequences of DNA methylation on epigenetic programming, and fetal growth and development.

References

[1] Bruinse HW, van den Berg H. Changes of some vitamin levels during and after normal pregnancy. Eur J Obstet Gynecol Reprod Biol 1995; 61:31–7.

[2] Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993–1016.

[3] Oommen AM, Griffin JB, Sarath G, Zempleni J. Roles of nutrients in epigenetic events. J Nutr Biochem 2005;16:74–7.

[4] Kim YI, Pogribny IP, Basnakian AG, Miller JW, Selhub J, James SJ, et al. Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. Am J Clin Nutr 1997;65:46–52.

[5] Van der Molen E, Verbruggen B, Novakova L, Eskes TK, Monnens LA, Blom HJ. Hyperhomocysteinaemia and other thrombotic risk factors in women with placental vasculopathy. Br J Obstet Gynaecol 2000;107:785–91.

[6] Hankey J, Eikelboom JW. Homocysteine and vascular disease. Lancet 1999;354:407–13.

[7] Rajkovic A, Catalano PM, Malinow MR. Elevated homocysteine levels with preeclampsia. Obstet Gynecol 1997;90:168–71.

[8] Wouters MGAJ, Boers GHJ, Blom HJ, Trijbels FJM, Thomas CMG, Borm GF, et al. Hyperhomocysteinemia: a risk factor in women with unexplained recurrent early pregnancy loss. Fertil Steril 1993;60: 820–5.

[9] Burke G, Robinson K, Refsum H, Drumm J, Graham I. Intrauterine growth retardation, perinatal death, and maternal homocysteine levels. N Engl J Med 1992;326:69–70.

[10] Goddijn-Wessel TA, Wouters MG, van de Molen EF, Spuijbroek MD, Steegers-Theunissen RP, Blom HJ, et al. Hyperhomocysteinemia: a risk factor for placental abruption or infarction. Eur J Obstet Gynecol Reprod Biol 1996;66:23–9.

[11] Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, et al. Homocysteine metabolism in pregnancies complicated by neural-tube defects. Lancet 1995;345:149–51.

[12] Ehrlich M. Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem 2003;88: 899–910.

[13] MacLennan NK, James SJ, Melnyk S, Piroozi A, Jernigan S, Hsu JL, et al. Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. Physiol Genomics 2004;18:43–50.

[14] Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 2003; 23:5293–300.

[15] Lee H, Kim J-M, Kim HJ, Lee I, Chang N. Folic acid supplementation can reduce the endothelial damage in rat brain microvasculature due to hyperhomocysteinemia. J Nutr 2005;135:544–8.

[16] Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. J Chromatogr 1987;422:43–52.

[17] Hyun TS, Han YH, Lim EY. Blood folate level determined by a microplate reader and folate intake measured by a weighed food record. Korean J Community Nutr 1999;4:512–20.

[18] Wang W, Kramer PM, Yang S, Pereira MA, Tao L. Reversed-phase high-performance liquid chromatography procedure for the simultaneous determination of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine in mouse liver and the effect of methionine on their concentrations. J Chromatogr 2001;B762:59–65.

[19] Kim HH, Park JH, Seong KS, Lee S. Determining the global DNA methylation status of rat according to the ID repetitive elements. Electrophoresis 2007;28:3854–61.

[20] Sibani S, Melnyk S, Pogribny IP, Wang W, Hiou-Tim F, Deng L, et al. Studies of methionine cycle intermediates (SAM, SAH), DNA methylation and the impact of folate deficiency on tumor numbers in Min mice. Carcinogenesis 2002;23:61–5.

[21] Wainfan E, Dixik M, Stender M, Christman JK. Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. Cancer Res 1989;49:4094–7.

[22] Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217–46.

[23] Caudill MA, Wang JC, Melnyk S, Pogribny IP, Jernigan S, Collins MD, et al. Intracellular *S*-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. J Nutr 2001;131: 2811–8.

[24] Owen EP, Human L, Carolissen AA, Harley EH, Odendaal HJ. Hyperhomocysteinemia — a risk factor for abruption placentae. J Inherit Metab Dis 1997;20:359–62.

[25] Mathews F, Yudkin P, Neil A. Influence of maternal nutrition on outcome of pregnancy: prospective cohort study. BMJ 1999;319: 339–43.

[26] Steegers-Theunissen RP, Smith SC, Steegers EA, Guilbert LJ, Baker PN. Folate affects apoptosis in human trophoblastic cells. BJOG 2000; 107:1513–7.

[27] Antony AC. In utero physiology: role of folic acid in nutrient delivery and fetal development. Am J Clin Nutr 2007;85:598S–603S.

[28] Rondo PH, Tomkins AM. Folate and intrauterine growth retardation. Ann Trop Paediatr 2000;20:253–8.

[29] Li E, Beswtor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 1992;69: 915–26.

[30] Matsuda M, Yasutomi M. Inhibition of cephalic neural tube closure by 5-azacytidine in neurulating rat embryos in vitro. Anat Embryol 1992; 185:217–23.