High-dose folic acid supplementation in rats: effects on gestation and the methionine cycle

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There is new evidence that a good folate status may play a critical role in the prevention of neural-tube defects and in lowering elevated homocysteine concentrations. This adequate folate status may be achieved through folic acid dietary supplementation. Folate is a water-soluble vitamin with a low potential toxicity. However, the possible consequences of long-term high-dose folic acid supplementation are unknown, especially those related to the methionine cycle, where folate participates as a substrate. With the aim of evaluating such possible effects, four groups of Wistar rats were classified on the basis of physiological status (virgin *v.* pregnant) and the experimental diet administered (folic-acid-supplemented, 40 mg/kg diet *v.* control, 2 mg folic acid/kg diet). Animals were fed on the diets for 3 weeks. Results showed that gestation outcome was adequate in both groups regardless of the dietary supplementation. However, there were reductions ($P < 0.001$) in body weight and vertex–coccyx length in fetuses from supplemented dams *v.* control animals. Folic acid administration also induced a higher ($P < 0.01$) S-adenosylmethionine : S-adenosylhomocysteine value due to increased S-adenosylmethionine synthesis ($P < 0.01$). However, hepatic DNA methylation and serum methionine concentrations remained unchanged. Serum homocysteine levels were reduced in supplemented dams ($P < 0.05$). Finally, pregnancy caused lower serum folate, vitamin B_6 and vitamin B_{12} levels ($P < 0.05$). Folic acid administration prevented the effect of pregnancy and raised folate levels in dams, but did not change levels of vitamins B_{12} and B_6. These new findings are discussed on the basis of potential benefits and risks of dietary folic acid supplementation.

Folate: Methionine cycle: Pregnancy: Toxicity

Folic acid has become very popular since the early 1990s, mainly due to the evidence that supplementation of the diet with the vitamin during the periconceptional period and in early pregnancy can reduce the incidence of congenital abnormalities, such as neural-tube defects (NTD) (MRC Vitamin Study Research Group, 1991; Czeizel & Dudas, 1992; Czeizel *et al.* 1996). In addition, there is now agreement that a good folate status is associated with the reduction of hyperhomocysteinaemia, recently confirmed as a new risk factor for cardiovascular disease (Loscalzo, 1996; Welch & Loscalzo, 1998).

Very recently, new dietary reference intakes for folate have been reported (Bailey, 1998). For the first time, the concept of dietary folate equivalents (DFE) differentiates naturally occurring food folate from synthetic folate (used for dietary supplementation and fortification) because of their different bioavailabilities. On this basis, the dietary reference intake for adults is established at 400 μg DFE/d and the recommendation for pregnancy has been raised to 600 μg DFE/d (Bailey, 1998). However, the minimum effective and safe dose of folic acid to prevent NTD or reduce high homocysteine levels still remains unknown.

In addition to its use as a supplement, it has been suggested that food fortification with folic acid could lead to an adequate folate status for all women of child-bearing age, thus reducing the overall incidence of NTD (Department of Health, 1992; Health Council/Food and Nutrition Council, 1992, 1993; Food and Drug Administration, 1993; Center for Disease Control, 1991). Unfortunately, there is not much information available about the potential risks associated with the proposed 'new pharmacological' actions of this vitamin. The most well-known risk of exposure to high doses of folate is the possible masking of cobalamin deficiency in pernicious anaemia (Lachance, 1998), since

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folate supplementation may reduce haematological signals but not neurological disease from a prolonged cobalamin deficiency. In addition, Hook & Czeizel (1997) recalled that in the only randomized occurrence study of dietary folic acid supplementation in women, there was not only a diminished prevalence of birth defects, but also a higher prevalence (16 % relative increase) of spontaneous abortion (embryonic and early fetal death). According to these authors, this raises several questions, including the hypothesis that folic-acid-induced terathanasia could explain the protective effect of folate with regard to birth defects. Hence, although folic acid is generally well tolerated, dietary supplementation could induce adverse effects that we are unaware of at present.

Folate is one of the essential effectors involved in the nutritional regulation of the methionine cycle (Finkelstein, 1990). Many studies have reported that a deficiency in folate clearly leads to an impaired functioning of this cycle (Balaghi *et al.* 1992, 1993; Selhub & Rosenberg, 1996). However, since few studies have been carried out on high-dose folic acid administration, mostly due to ethical reasons, we ignore at present to what extent an excess of folic acid could interfere with the regulatory mechanism of the methionine cycle. These possible implications could be quite relevant in a physiological situation of nutritional stress such as pregnancy.

On the basis of all these issues, the present study was undertaken to determine the effects of long-term dietary supplementation on gestational and biochemical markers related to the methionine cycle, in pregnant and virgin rats. The level of supplementation chosen was 40 mg folic acid/kg diet, i.e. twenty times the level considered adequate for pregnant rats (2 mg folic acid/kg diet).

Methods

Animals and diets

Thirty-eight female Wistar rats (with an initial weight of approximately 180 g; Animal Service, Universidad San Pablo-CEU, Madrid, Spain) were classified into four different groups on the basis of their physiological status (pregnant *v.* virgin) and the experimental diet administered (folic-acid-supplemented *v.* control): supplemented dams were fed on the folic-acid-enriched diet (40 mg folic acid/kg diet); control dams were fed the control diet (2 mg folic acid/kg diet); a group of virgin rats was also fed on the folic-acid-supplemented diet and, finally, virgin rats were also fed on the control diet. Animals were individually housed in metabolic cages especially designed for pregnant rats, and were maintained in a room with a 12 h light/dark cycle, 20–23°, and with an appropriate ventilation system.

Both diets were adjusted to rat requirements (National Research Council, 1995), and were based on the pure amino acid diet (170 g amino acid/kg, Dyets, Bethlehem, PA, USA) described by Walzem & Clifford (1988). This is the most reliable system for studying the exclusive effect of dietary folic acid, without confounding factors, as demonstrated in several previous studies (Walzem & Clifford, 1988; Clifford *et al.* 1989, 1993; Varela-Moreiras & Selhub, 1992; Varela-Moreiras *et al.* 1995*b*; Alonso-Aperte & Varela-Moreiras, 1996).

All experiments were undertaken according to *Directional Guides Related to Animal Housing and Care* (European Community Council, 1986).

Experimental design

Rats were fed on their respective folic-acid-supplemented or control diet for 3 weeks, including a complete pregnancy in dams. All groups of animals were given free access to food and water. Intake and weight were assessed every 48–72 h, and the food efficiency ratio (FER) was determined. On day 21 of the experiment, animals were lightly anaesthetized with CO_2 and killed by decapitation. Embryonic development was evaluated by measuring fetal vertex-coccyx length and weight, and total number in the litter. Whole blood was collected and the serum was separated by centrifugation and kept at −20° until analysed. Livers were promptly removed, frozen in liquid N_2 and stored at −70° for further analyses.

S-adenosylmethionine and S-adenosylhomocysteine

Hepatic S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels were determined by HPLC according to the method described by Fell *et al.* (1985), with some modifications (Miller *et al.* 1994). Portions of frozen liver (about 100 mg) were homogenized in four volumes of 0·4 M-$HClO_4$, and then centrifuged at 10 000 *g*, 4°, for 10 min. The clear supernatant fractions were removed, filtered, and appropriate samples were analysed for SAM and SAH.

DNA methylation

The capacity of hepatic DNA preparations to serve as methyl group acceptors was determined using the method of Christman *et al.* (1980) which was modified by replacing the DNA methylase from Friend erythroleukaemia cells with SssI methylase from *E. coli* (New England Biolabs, Beverly, MA, USA). Briefly, DNA (2 µg), SssI (4 U), and [³H-methyl]SAM (185 kBq) in 20 µl buffer containing 50 mM-NaCl, 10 mM-Tris-HCl, pH 8·0, and 10 mM-EDTA were incubated for 3 h at 37°. The reaction was stopped by heating the mixture for 20 min at 65°. The mixture was then applied onto a disk of Whatman DE-81 paper (Whatman International Ltd, Maidstone, Kent, UK) and soaked in 50 ml 0·363 M-NaH_2PO_4 for 45 min. Radioactivity retained on the disk was determined by scintillation counting using a non-aqueous scintillation fluor. The amount of radioactivity bound to a filter from an incubation mixture lacking only DNA was used as background and was subtracted from the values obtained with mixtures containing DNA. Because this is an inverse assay, a higher incorporation of [³H]methyl groups into DNA in the *in vitro* assay indicates a diminished *in vivo* methylation of DNA.

Methionine

Portions of serum samples (400 µl) were deproteinized by ultrafiltration and analysed using a Beckman System 6300 High Performance Amino Acid Analyser (Beckman Instruments, Palo Alto, CA, USA), according to the modifications by Andersson *et al.* (1989).

Homocysteine

Serum homocysteine levels were determined using a Chromsystems Reagent Kit for HPLC analysis of homocysteine in serum (Chromsystems, München, Germany), which uses a simple isocratic HPLC system with an attached fluorescence detector (λ excitation wavelength 385 nm; emission wavelength 515 nm).

Folate and vitamin B_{12}

Serum folate and vitamin B_{12} levels were determined by chemiluminescence, using a Ciba-Corning reagent kit for folate and B_{12} determinations, with an automated chemiluminescence system (Ciba-Corning ACS^{TM}, Medfield, MA, USA).

Vitamin B_6

Serum vitamin B_6 levels were determined using a Chromsystems reagent kit for HPLC analysis of vitamin B_6 in serum, which uses a simple isocratic HPLC system with an attached fluorescence detector (excitation wavelength 300 nm; emission wavelength 400 nm).

Statistics

The data were statistically analysed by a two-way ANOVA. When ANOVA resulted in differences, multiple comparisons between means were studied by the Tukey test. All values are expressed as means with their standard errors. Differences were considered significant at $P < 0.05$ (Systat Version 5.0, Systat Inc., Chicago, IL, USA).

Results

General nutritional status

Table 1 shows body-weight gain and food intake of the animals during the 21 d under experimental conditions. The FER was calculated as the ratio between daily body-weight gain and daily dietary intake. Pregnancy, regardless of dietary folic acid level, resulted in a significantly higher FER ($P \leq 0.001$) than that seen in virgin groups, as a direct consequence of higher weight increase ($P < 0.001$). Dietary folate supplementation did not alter the FER values significantly.

Gestation outcome

The main findings may be observed in Table 2. On the basis of the number of live fetuses, gestational development was considered adequate and similar in both groups. However, there were significant reductions ($P < 0.001$) in body weight and vertex-coccyx length in fetuses from dams supplemented with folic acid, when compared with fetuses from control dams.

Maternal biochemical variables

With respect to the main biomarkers involved in the functioning of the methionine–methylation cycle (Table 3), the

Table 1. Body-weight gain, food intake and food efficiency ratio (FER) in pregnant and virgin Wistar rats fed on folic-acid-supplemented (40 mg/kg diet) or control (2 mg/kg) diets for 21 d

(Mean values with their standard errors)

Group	n	Δ Body weight (g/d)		Food intake (g/d)		FER†	
		Mean	SEM	Mean	SEM	Mean	SEM
SUP DAMS	11	5·7***	0·44	20·7*	0·56	0·26***	0·01
CON DAMS	9	6·5***	0·82	22·6	0·52	0·29***	0·01
SUP VIRGIN	8	2·5	0·19	24·4	1·70	0·10	0·01
CON VIRGIN	10	2·7	0·20	24·3	0·94	0·11	0·00

SUP DAMS, supplemented dams; CON DAMS, control dams; SUP VIRGIN, supplemented virgin; CON VIRGIN, control virgin.
Mean values were significantly different from those for the virgin groups: *$P < 0.05$, ***$P \leq 0.001$.
† Δ Body weight/food intake.

Table 2. Gestation outcome in Wistar rat dams fed on folic-acid-supplemented (40 mg/kg diet) or control (2 mg/kg) diets

(Mean values with their standard errors)

Group	Dams n	Live fetuses/litter (n)		Fetal body weight (g)		Fetal vertex-coccyx length (mm)	
		Mean	SEM	Mean	SEM	Mean	SEM
SUP DAMS	11	11·4	1·16	3·15***	0·10	35***	0·5
CON DAMS	9	11·6	0·74	3·49	0·22	37	0·9

SUP DAMS, supplemented dams; CON DAMS, control dams.
Mean values were significantly different from those of the control group: ***$P < 0.001$.

Table 3. Hepatic S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) concentrations, values for methylation ratio (SAM : SAH) and serum methionine and homocysteine concentrations in pregnant and virgin Wistar rats fed on folic-acid-supplemented (40 mg/kg diet) or control (2 mg/kg) diets for 21 d

(Mean values with their standard errors)

Group	n	SAM (nmol/g) Mean	SEM	SAH (nmol/g) Mean	SEM	SAM : SAH Mean	SEM	Methionine (nmol/ml) Mean	SEM	Homocysteine (μmol/l) Mean	SEM
SUP DAMS	11	86·28**	3·31	40·25	3·64	2·31**	0·23	93·0	3·80	12·5†	1·1
CON DAMS	9	71·18	4·28	41·91	5·64	1·88	0·20	104·7	7·41	14·9	0·8
SUP VIRGIN	8	92·93**	2·64	38·49	2·05	2·45**	0·13	96·7	6·81	16·4	1·2
CON VIRGIN	10	78·96	2·89	40·84	2·12	1·97	0·12	109·3	9·00	17·6	1·2

SUP DAMS, supplemented dams; CON DAMS, control dams; SUP VIRGIN, supplemented virgin; CON VIRGIN, control virgin.
Mean values were significantly different from those for the corresponding control group: **$P < 0.01$.
Mean values were significantly different from those for the control virgin group: † $P < 0.05$.

two folic-acid-supplemented groups had significantly higher hepatic SAM concentrations ($P < 0.01$), whereas SAH values remained unchanged. In consequence, the value of the SAM : SAH concentration ratio, also known as 'methylation ratio', was significantly higher ($P < 0.01$) when animals were receiving the supplemented diet. Despite these results, hepatic DNA global methylation did not show significant intergroup differences, maybe due to a large intragroup variability (Fig. 1).

Serum methionine levels were slightly reduced in both groups of animals receiving folate, although differences were not significant (Table 3). Serum homocysteine concentration was significantly reduced in dams receiving folate ($P < 0.05$) when compared with control animals (Table 3).
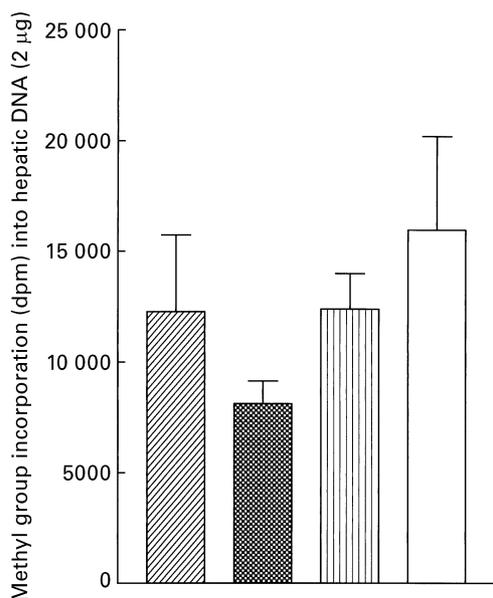
Levels of vitamins involved in the nutritional regulation of the methylation cycle were also examined (Table 4). As expected, supplementation with folic acid led to a significant increase in serum folate concentrations ($P < 0.05$) in both dams and virgin animals. Conversely, pregnancy itself caused lower serum folate levels ($P < 0.05$) in dams. This effect was partially prevented in the dams receiving folate, since values for folate serum concentrations in these dams were similar to those for control virgin animals. Serum vitamin B_{12} levels were also markedly reduced in pregnancy ($P < 0.001$). This same pattern was observed for vitamin B_6, since dams showed significantly lower serum levels ($P < 0.05$) when compared with virgin rats. Folic acid administration did not influence vitamin B_6 or B_{12} concentrations significantly.

Discussion

Dietary supplementation with folic acid is being widely recommended nowadays to prevent NTD during pregnancy (MRC Vitamin Study Research Group, 1991; Department of Health and Human Services, 1992; Center for Disease Control, 1991) and to reduce high homocysteine levels (Ubbink, 1994). The vitamin is generally considered as safe, although we do not have enough information about its possible interrelationships with other micronutrients or how it may regulate or deregulate critical metabolic cycles, such as the methionine cycle. Therefore, the present study was carried out to examine the effects of long-term high-dose dietary supplementation with folic acid on the methionine cycle and other nutritional markers in both pregnant and virgin Wistar rats.

Folic acid administration was achieved using a diet enriched with 40 mg folic acid/kg. This level of supplementation corresponds to twenty times the folic acid level in the control diet, i.e. 2 mg folic acid/kg diet, considered adequate for pregnant Wistar rats (National Research Council, 1995; Alonso-Aperte, 1997). For man, the highest dose of folate issued is the recommendation to high-risk women to prevent NTD recurrence, i.e. 4 mg/d (Center for Disease Control, 1991; Department of Health and Human Services, 1992). This dose is also twenty times the most frequently recommended level of dietary folate intake for non-pregnant women, i.e. 200 μg/d (de Bree *et al.* 1997), although recent research has raised the recommendation to 400 μg

Fig. 1. Global DNA methylation in liver from pregnant or virgin Wistar rats fed on folic-acid-supplemented (40 mg/kg diet) or control (2 mg/kg) diets for 21 d. Results are expressed as methyl group (disintegrations/min; dpm) incorporation into DNA (2 μg) isolated from rat liver. As an inverse assay, a greater *in vitro* incorporation of methyl groups indicates a lower degree of *in vivo* DNA methylation, and vice versa. Values are means for eight to eleven rats, with their standard errors represented by vertical bars. (▨), Supplemented dams (*n* 11); (▨), control dams (*n* 9); (▥), supplemented virgins (*n* 8); (□), control virgins (*n* 10).

Table 4. Serum folate, vitamin B_{12} and vitamin B_6 levels in pregnant and virgin Wistar rats fed on folic-acid-supplemented (40 mg/kg diet) or control (2 mg/kg) diets for 21 d

(Mean values with their standard errors)

Group	n	Folate (ng/ml)		Vitamin B_{12} (ng/ml)		Vitamin B_6 (µg/l)	
		Mean	SEM	Mean	SEM	Mean	SEM
SUP DAMS	11	27.8*	2.9	1002‡	72.3	101.0†	7.3
CON DAMS	9	4.1‡	1.1	1079‡	42.5	103.9†	11.8
SUP VIRGIN	8	43.4*	3.8	2088	49.2	154.5	17.7
CON VIRGIN	10	23.4	3.8	1913	71.6	175.0	23.5

SUP DAMS, supplemented dams; CON DAMS, control dams; SUP VIRGIN, supplemented virgin; CON VIRGIN, control virgin.

Mean values were significantly different from those for the corresponding control group: $*P < 0.05$.

Mean values were significantly different from those for the virgin groups: $† P < 0.05$.

Mean values were significantly different from those for the control virgin group: $‡ P < 0.05$.

DFE/d (Bailey, 1998). Therefore, the level of supplementation in the present study can be considered high, and above the usual folate recommendation issued to all women in order to prevent occurrence of NTD.

When general nutritional status was studied, we observed that supplementation with folic acid did not affect normal growth in rats. This is in accordance with other studies in which folate-enriched diets did not improve growth response (Clifford *et al.* 1993; Alonso-Aperte, 1997). As expected, pregnant rats had a higher FER when compared with virgin rats, but folic acid level in the diet did not influence FER. This indicates an adequate gestational development with both folic acid levels in the diet. There are no other comparable studies at present using our dietary folic acid level and experimental design. Gestational development was also considered adequate in both groups according to the number of live fetuses. Despite this acceptable gestational development, there were significant reductions in body weight and vertex-coccyx length in fetuses from dams fed on the diet supplemented with high doses of folic acid. The consequences of these interesting observations are unknown at this time, and it will be necessary to study post-partum evolution of these low weight and length new-born fetuses, especially up to another critical period such as lactation.

With respect to the effect on the methylation cycle, both groups of animals receiving folate supplement had significantly higher hepatic SAM concentrations, regardless of the physiological status. A positive effect of folate administration on SAM levels has also been described by Balaghi *et al.* (1993). Conversely, SAH values remained unchanged. In consequence, the value for the SAM : SAH concentration ratio, also known as the methylation ratio, was significantly higher in both dams and virgin rats fed on the folate-enriched diet. A low methylation ratio has been associated with inactivation of transmethylation reactions (Hoffman *et al.* 1979), therefore a higher SAM availability could facilitate these reactions. In the present study, nevertheless, hepatic DNA global methylation was not significantly affected either by the diet used or the physiological status. The values obtained are within the same range of control values previously obtained by Varela-Moreiras *et al.* (1995*a*) in hepatic injury studies. Although SAM is involved in many other methylation reactions, our data on DNA

methylation and SAH concentration do not show a higher transmethylation activity due to folate administration.

The possible consequences of elevated hepatic SAM concentrations are at present unknown. SAM is essential in many transmethylation reactions and, therefore, in development and growth (Finkelstein, 1990), but its specific role or possible effects on gestation have not been studied before. A higher SAM availability could have a positive effect on development since DNA hypomethylation and hypomethylation of contractile and basic myelin proteins have been proposed as mechanisms of teratogenesis (Coelho & Klein, 1990; Li *et al.* 1992; Mills *et al.* 1996). Conversely, a higher SAM synthesis could induce a negative effect due to a lowering in methionine concentration. In fact, in our study both groups fed on the folic-acid-supplemented diet showed lower methionine concentrations. Although these results were not significantly different, they might actually show a relevant tendency. Methionine may be an essential amino acid in development, an effect that has been observed in animal models (Coelho *et al.* 1989; Coelho & Klein, 1990; Alonso-Aperte *et al.* 1999).

A reduction of maternal serum homocysteine levels has been described during normal pregnancies in healthy women, probably due to either haemodilution or a metabolic adaptation to an increased requirement for methionine by the fetus (Steegers-Theunissen *et al.* 1994). With respect to this issue, our results do not show a reduction in homocysteine levels due to gestation, since concentration of the amino acid in control dams did not differ significantly from the value in control virgins. The capacity of dietary supplementation with folate to reduce homocysteine levels has been widely demonstrated in human subjects (Ubbink, 1994; Mills *et al.* 1996; Kang, 1996; Santhosh Kumar *et al.* 1997; Dierkes *et al.* 1998; Malinow *et al.* 1998), as well as in the rat (Alonso-Aperte, 1997) and rat embryos (Van Aerts *et al.* 1994). In our present study, high-dose dietary folic acid was only capable of reducing homocysteine levels in dams. This effect of folic acid during gestation is quite interesting since maternal hyperhomocysteinaemia could be causally related to abnormal embryo development (Steegers-Theunissen *et al.* 1994; Mills *et al.* 1996).

Folate, vitamin B_{12} and vitamin B_6 act as substrate and

cofactors respectively in the methylation cycle, and thus they are the only micronutrients that participate in the nutritional regulation of this cycle. Several studies in rats (Clifford et al. 1993; Alonso-Aperte, 1997), and more recently in human subjects (Malinow et al. 1998), have reported that serum folate levels are directly related to dietary folate intake. Therefore, as expected, dietary supplementation with folic acid led to an elevation in the serum levels of the vitamin. Conversely, vitamin B_{12} and vitamin B_6 levels were not affected by folic acid administration. This same pattern has been observed in previous research which does not show an alteration in vitamin B_{12} or B_6 levels when feeding rats with folate-enriched diets (Alonso-Aperte, 1997). Pregnancy itself definitely caused lower serum folate levels in the control dams. This stressing effect of gestation on serum vitamin levels was also marked for vitamins B_{12} and B_6, thus concentrations were significantly reduced. This effect may be indicative of much higher utilization of these vitamins in tissues as has been previously proposed (McNulty et al. 1993; McPartlin et al. 1993). Regarding this issue, it is interesting to note that folic acid administration prevented the effect of gestation on serum folate, since the concentration of the vitamin in supplemented dams was significantly higher than in control dams and the value was similar to that of control virgin rats. These results prove once more the efficacy of dietary supplementation with folate to prevent folate deficiency during pregnancy, an application that has been, for a considerable length of time, recommended to all pregnant women in the second trimester of pregnancy. On the other hand, the lowering effect of gestation on serum vitamin levels shows that pregnancy could predispose to vitamin B_{12} deficiency. This is worrying since the only currently recognized risk of folate excess is the masking of vitamin B_{12} deficiency (Lachance, 1998).

In conclusion, high-dose dietary supplementation with folic acid (40 mg folic acid/kg diet) in rats, (a) induces a higher synthesis of SAM, and the potential positive or negative effect of a higher SAM availability remains unknown; (b) reduces serum homocysteine concentration in gestation, which could be considered as beneficial; (c) reduces body weight and length in fetuses. The consequences of this effect need to be further studied.

Therefore, an urgent requirement for more studies to further develop or refute these effects of folate is indicated, since they may contribute to a better understanding of the potential benefits and risks of high folate levels and the mechanisms involved. However, the results obtained in the present study are due to high-dose folate administration in rats and, therefore, the effects may not be relevant at this point with usual supplementary folate levels in human subjects.

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