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Is calcium levofolinate pentahydrate more effective than folic acid in young healthy women before conception?

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Summary

Purpose of investigation: To assess whether l,5-formyl-tetrahydrofolic (levofolinic acid, LV) is better than folic acid (FA) to increase serum folate and to reduce homocysteine in healthy young women during reproductive age. *Materials and Methods:* Folate deficiency is related to a number of pregnancy complications. FA is a synthetic compound that has no biological functions unless it is reduced to tetrahydrofolate. Supplementation of an active form such as LV, could be a better alternative. The authors performed an 8-week 1:1 randomized, open label clinical trial in 40 healthy women aged between 18-40 years, with daily 4 mg LV or 400 mcg FA supplementation. *Results:* Serum folate were increased after LV or FA supplementation, as compared to baseline, without any significant differences within the two groups ($p = 0.8$). Homocysteine levels were reduced in both groups, by 31.6% ($p < 0.001$) and 26.3% ($p < 0.001$) respectively, however the reduction was much relevant in the LV group ($p < 0.001$). *Conclusions:* Supplementation with LV seems to be more effective to reduce homocysteine levels.

Key words: Folic acid; Levofolinic acid; Homocysteine; Folate.

Introduction

Folate are a group of water soluble coenzymes, belonging to the vitamin B group. They play an important role in amino acid metabolism, purine and pyrimidine synthesis, and methylation of a large number of nucleic acids, proteins, and lipids. One of the most important folate-requiring process is the homocysteine/methionine cycle. Homocysteine is a non-essential amino acid, whose only source in the human body is the catabolism of methionine, with folate acting as methyl donors [1]. This process requires an adequate supply of folate and the normal activity of the enzyme methylenetetrahydrofolate reductase (MTHFR).

Genetic abnormalities in the function of MTHFR may increase the values of total homocysteine in plasma. Carriers of the abnormal homozygous variant (T/T) of the C677T mutation in the gene encoding this enzyme produce a thermolabile variant with less functional activity, requiring a continuous supply of folate to avoid hyperhomocysteinemia [2].

Folate deficiency causes hyperhomocysteinemia and a wide number of diseases like anemia, depression and pregnancy complications. There is strong evidence that, together with a low maternal folate status, even a moderate hyperhomocysteinemia is associated with an increased risk of a wide spectrum of late pregnancy complications, such as preeclampsia, abruptio placentae, intrauterine growth retardation (IUGR), preterm birth and intrauterine fetal death [3-6], therefore homocysteine serum concentrations should be as low as possible during pregnancy.

The main sources of folate are green leafy plants. 5-methyl-tetrahydrofolate (5MTHF) is the active metabolite of folate cycle, and the most available folate form in plants, human plasma, and human whole blood, representing 95–98% of folate in serum or red blood cells (RBC) [7]. Folate-requiring metabolic processes are influenced by dietary folate intake. Food folate concentration is conditioned by storage and cooking modes, which can substantially reduce the original folate amount. For this reason it is possible to assume folate in the form of supplementation drugs, and nowadays more than 50 countries are promoting folate food fortifications. Folic acid (FA), the first synthetic compound, has not physiological function, it must be reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) to be able to enter the folate cycle, and it has a tolerable upper intake level (UL) of 1 mg/day [8]. Levofolinic acid (LV), is an active form of folate naturally found in foods and metabolically active after absorption [9]. The conversion of LV to 5MTHF does not require MTHFR and therefore it is not altered in people with the T/T genotype of this enzyme [10]. In contrast to FA, LV has no UL [8].

Due to high metabolism and fetal needs, women have

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higher requirements for folate during pregnancy and lactations. Dietary folate is protective, but it can be insufficient because the recommendations are often not literally followed or because the bioavailability of food folate is variable. An optimal folate status must be achieved before conception and maintained throughout pregnancy and breastfeeding. For adults ≥ 19-years-old, the recommended dietary allowance (RDA) is 400 mcg/d of dietary folate equivalents (DFE) [11]. During pregnancy, there is an active transport to the child via the placenta or the milk, and maternal folate serum levels are the main determinant of folate status in neonates, suggesting that any improvement of maternal folate serum levels can ensure a better folate status in the fetus and the newborns [12]. 5MTHF is the main folate form in cord blood representing around the 90% of total folate [12], hence a supplementation with LV during pregnancy could provide an immediate source for folate to be transported to the fetus.

Although these observations suggest potential advantages of LV over folic acid, there are only a few studies on the effect of LV administration on homocysteine and serum folate concentrations in women of childbearing age. The aim of this preliminary study was to evaluate the fasting serum concentration of folate and homocysteine after an 8-week treatment with either 4 mg of LV or 400 mcg of FA in healthy young women during reproductive age.

Materials and Methods

From July to September 2016, 40 healthy women referred to the Obstetrics and Gynecology Department, University Hospital Policlinico G. Rodolico, Catania, Italy, were prospectively enrolled in the study. Healthy young women with adequate folate (6-10 ng/ml) and homocysteine (4-10 µmol/l) status, were eligible for participation. The main exclusion criteria were organic (i.e. liver, renal, cardiovascular disease) or mental disease, medical treatments interfering with folate metabolism (i.e. methotrexate, sulfasalazine, salicylic acid, antiepileptic drugs), previous history of congenital fetal defects, habitual abortion, abruption placentae, preeclampsia, stillbirth, pregnancy or lactation, abuse of alcohol or drugs, smoking, current or recent consumption of folate, vitamins B12 and B6 or multivitamins, supplementation. Patient's characteristics are shown in Table 1. There were no differences in race, BMI or parity within the two groups. Participants underwent randomization 1:1, LV 4 mg (calcium levofolinate pentahydrate 4 mg) or FA 400 mcg (folic acid 400 mcg), for 8 weeks. All participants were advised not to use any other folate or vitamin supplements during the study. At the baseline and after the treatment period a fasting blood sample (12 hour overnight fast) was drawn in the morning to determine total plasma homocysteine and serum folate.

The study protocol was conformed to the ethical guidelines of the 1975 Helsinki Declaration and it was approved by the Institutional Review Board of the department. Written informed consent was obtained from each patient at the enrollment. The study was not advertised and no remuneration was offered.

Blood samples for the measurement of the serum folate were drawn from the median cubital vein into EDTA coated tubes, centrifuged at 4°C within 15 minutes (1000x g, 10 minutes) and stored at -80°C until analysis within three months after. Total plasma folate was measured using an immunoassay kit (intra-assay CV < 5.4%; interassay CV < 8.3%).

Table 1. — *Demographic characteristics*

	LV (n20)	FA (n20)	*p*
Age (years)	31.3 (DS 5.1)	31.6 (DS 4.6)	NS
BMI (kg/m^2)	23.6 (DS 4.5)	24.5 (DS 3.8)	NS
Race			
Caucasian	20	20	NS
Black	0	0	
Others	0	0	
MTHFR			NS
Polymorphism			
Normal	16	17	
C677T homozygote	2	1	
Heterozygote, others	2	2	

For total homocysteine assay, whole blood samples were collected on ice-cooled tubes containing K3 EDTA and centrifuged within 30 minutes at 2000× g for 10 minutes at 4°C; plasma aliquots were frozen at −80 °C until assayed. Plasma homocysteine concentration was determined by fluorescence polarization immunoassay (FPIA) (intra-assay and interassay CV less than 8%).

The authors used PCR amplification for the study of the C677T mutation of the MTHFR gene. The reaction conditions were as follows: initial denaturation at 95°C for 15 minutes and 30 subsequent cycles at denaturation at 94°C for 60 seconds, annealing at 61°C for 60 seconds and extension at 72°C for twp minutes. PCR product (173-bp) was subjected to restriction digestion with 1U of HinfI enzyme. DNA amplification and restriction analysis by HinfI were carried out as previously described [13].

Median values with interquartile ranges (25–75th percentiles) were displayed, since the changes in serum folate and homocysteine concentrations were not normally distributed. The response to treatment was calculated for each participant as the absolute difference and the percentage variation between homocysteine concentration and serum folate before and after treatment.

Kruskal–Wallis test was used to compare changes in homocysteine concentrations from baseline to follow-up (post-treatment, postT). Differences in pre-treatment (preT) serum levels among groups were derived through Mann-Whitney test. In the hypothesis tests, values of $p < 0.05$ were accepted as statistically significant. Statistical analysis was carried out using the Primer of Biostatistics statistical computer package.

Results

The demographic characteristics of the study population are shown in Table 1. The two groups did not differ significantly for age, body mass index or MTHFR polymorphism. No significant differences among groups were observed in concentrations of total homocysteine and folate status before treatment. The number of subjects with a polymorphism in the MTHFR gene was very little and did not allow any statistical study.

After 8 week folate supplementation, a statistically significant increase in serum folate concentration was observed in both groups as compared to baseline. Serum folate were increased from 8.98 ng/ml (CI 6.45- 11.01) to

Table 2. — *Folate serum level pre and post treatment with 4 mg LV or 4mg FA.*

Treatment group	Pre-treatment serum folate (ng/mL) *	Post-treatment serum folate (ng/ml) *	Percentage of change after treatment	Intra-group "*p*" **
Levofolinic acid (LV)	8.98 (CI 6.45- 11.01)	13.19 (CI 10.77- 15.55)	+ 46.7%	$p < 0.001$
Folic acid (FA)	9.67 (CI 7.96- 10.93)	13.62 (CI 11.45- 15.79)	+ 47.7%	$p < 0.001$
Intergroup "*p*"***	$p < 0.9$	$p < 0.6$		

Data are presented as geometric means (95% CI); ** intra-group differences in pre- and post-treatment folate serum levels, through Wilcoxon's test; * inter group p-values derived for differences in median folate change after treatment,through Mann–Whitney's test.*

Table 3. — *Homocysteine serum levels pre and post treatment with 4 mg LV or 4mg FA.*

Treatment group	Pre-treatment Homocysteinemia (μmol/L)*	Post-treatment Homocysteinemia (μmol/L) *	Percentage of change after treatment	Intra group "p" **
Levofolinic acid (LV)	10.63 (CI 9.21- 12.05)	7.28 (CI 6.15- 8.41)	- 31,6%	$p < 0.001$
Folic acid (FA)	10.99 (CI 9.35- 12.63)	8.10 (CI 6.23- 9.97)	- 26,3%	$p < 0.001$
Intergroup "p"***	NS	$p < 0.001$		

Data are presented as geometric means (95% CI); ** intra-group differences in pre- and post-treatment homocysteine serum levels, through Wilcoxon's test; * inter-group p-values derived for differences in median homocysteine change after treatment,through Mann–Whitney's test.*

13.19 ng/ml (CI 10.77- 15.55) after 4 mg LV oral supplementation ($p<0.001$) and from 9.67 ng/ml (CI 7.96- 10.93) to 13.62 ng/ml (CI 11.45- 15.79) 400 mcg FA supplementation ($p<0.001$). There was no difference in the preT and postT folate serum levels within the two groups (preT difference inter groups not significant NS; posT difference inter groups NS) and therefore both supplementation drugs showed the same effectiveness (Table 2).

Hmocysteine concentrations were reduced after 4 mg LV or 400 mcg FA 8 week supplementation by 31.6% ($p < 0.001$) and 26.3% ($p < 0.001$) respectively, as compared to baseline levels (Table 3). However the reduction was much relevant in the LV group with a statistically significant difference across treatments ($p < 0.001$).

Discussion

Low maternal folate status as well as hyperhomocysteinemia are related to a number of pregnancy complications such as neural tube defects (NTDs), preeclampsia, IUGR and poor pregnancy outcome. The effective treatment to reduce homocysteine serum concentrations is folate administration, alone or in combination with B6 and/or B12 vitamins, even in people who do not have a clinically evident vitamin deficiency [14, 15]. An optimal folate status must be achieved before conception and then maintained throughout pregnancy and lactations, when dietary folate may be insufficient to reach a protective folate status. Therefore supplemental folate are highly recommended, and because of the limited time window for prevention, young women should have a daily supplementation of at least 400 mcg of folate starting 4 weeks before conception [16].

A recent meta-analysis quantified the dose-response relationship between folate intake (dietary folate plus FA) and folate biomarkers in young pregnant and lactating women, showing that during these physiological periods, maintaining maternal folate biomarkers at a given level is more difficult [17]. During pregnancy, there is an active transport to the child via the placenta or the milk, and maternal folate serum levels are the main determinant of folate status in neonates, suggesting that any improvement of maternal folate serum levels can ensure a better folate status in the fetus and the newborns [12]. 5MTHF is the main folate form in cord blood representing around the 90% of total folate [12], hence a supplementation with LV during pregnancy could provide an immediate source of folate for the fetus.

Dietary folate is protective, but it can be insufficient particularly during periods of high demand. LV is an active form of folate naturally found in foods and metabolically active after absorption [9]. Pharmacokinetic studies following oral administration of LV indicate that it is rapidly absorbed and metabolized by the mucosa of the small intestine and liver to 5MTHF [18], so that 90% of the increase in peripheral blood folate after LV oral supplementation corresponds to 5MTHF [19]. The conversion of LV to 5MTHF does not requires MTHFR and therefore it is not altered in people with the T/T genotype of this enzyme [12].

The present pilot study demonstrates that oral administration of 4 mg a day of LV for 8 weeks, in healthy women of reproductive age, decreases serum homocysteine levels better than FA, and it has the same effectiveness to increase serum folate. It was is quite difficult to compare the present results with previous studies. The studies conducted so far have administered FA or 5MTHF using very variable doses and duration of treatment and in most of cases folate were given in combination with other vitamins. The populations studied are very inhomogeneous regarding age,

polymorphism of MTHFR, medical history, and fertility status [20-23]. Furthermore there are only a few studies using LV as supplemental drug for women in childbearing age [24].

Pre- and post-treatment changes in fasting serum folate and homocysteine concentrations were the primary variables of interest of this study. The fasting serum concentration of folate is a good indicator of folate status, particularly useful as an early marker of folate depletion or repletion after dietary modification or supplementation, although it may fluctuate if folate intake is not constant. Compared to serum folate the concentration of RBC folate is an indicator for folate storage, and it takes longer than serum folate to reach a steady state after folate supplementation (approximately 40 weeks) [25]. Nevertheless serum folate is the strongest negative determinant of homocysteine concentrations in pregnant women [26]. For these reasons the present authors decided to take in account fasting serum folate rather than RBC folate as a marker of treatment response.

During the study period, homocysteine serum levels were reduced by 31.6% and serum folate was increased by 46.7% after the administration of 4 mg LV daily. These results demonstrate that 8 weeks of daily 4 mg LV supplementation are sufficient to decrease homocysteine values and improve folate status, in healthy young women before conception. If compared with 400 mcg FA supplementation, LV seems to more effective to reduce homocysteine serum concentration (7.28 mmol/L [CI 6.15- 8.41] *vs.* 8.10 mmol/L [CI 6.23- 9.97], $p < 0.001$). The observation that LV can improve homocysteine and folate serum concentrations even better than FA, leads to the assumption that LV supplementation could protect against a number of birth defects like NTDs congenital heart defects (CHDs), orofacial clefts, and pregnancy complications like IUGR, preterm birth or abortion [16, 27-31].

The polymorphism for MTHFR gene is found in ≈10–22% of the European population. Carriers of the abnormal homozygous variant (T/T) of the C677T mutation have higher homocysteine concentrations, lower folate serum levels [32], and show a weak response to FA supplementation together with an increased risk for NTDs [33, 34]. The incidence of MTHFR gene polymorphism in the present study population is similar to the estimated for Caucasian people, and it was 20% in the LV group and 15% in the FA group. Only two subjects out of 40 had homozygous variant (T/T), then the authors could not make any statistical conclusions about this mutation in their study population. This was one of the larger limitations of this study, together with the small number of the subject enrolled. Another limitation of this trial was the absence of a placebo group. As the health authorities and professional organizations in the authors' country recommend folate supplementation before conception, as was the case of the participants, a placebo-controlled study was not ethically possible.

Conclusions

Supplementation with LV and FA in young women of reproductive age reached a similar result in rising folate serum levels after 8 weeks of treatment; nevertheless LV seems to be more effective to reduce homocysteinemia.

Although there is a lack of clinical trials, there are a few studies, showing that LV is at least as effective as FA to improve folate biomarkers. FA can prevent a wide number of fetal complications by increasing serum folate level and decreasing homocysteine. LV can effectively increase serum folate and decrease homocysteine concentrations. Therefore, supplementing with LV against pregnancy complications seems to be rational. In the present authors' opinion, they do not have any reason to assume that a randomized controlled trial is needed before recommending LV to prevent pregnancy complications.

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