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# Rapid Improvement with Crystal form C of L-5-Methyltetrahydrofolate Calcium Salt in Maternal Women with Folate Deficiency: A Pilot Study

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***Abstract***

***Background:*** *For women of reproductive age, a population-level red blood cell (RBC) folate concentration below the threshold 906 nmol/L or 400 ng/mL indicates suboptimal neural tube defect (NTD) prevention. Currently, there is a lack of evidence to support whether L-5-methyltetrahydrofolate (L-5-MTHF) as a new source of folate supplementation is sufficient to raise RBC folate concentrations to a level associated with a reduced risk of a NTD-affected pregnancy.*

***Methods:*** *This study was a single-arm pilot study. 33 pregnant women aged 25 to 40 with folate deficiency were treated with L-5-MTHF for 8 weeks between November 2023 and September 2024. The primary outcome is RBC folate concentrations.*

***Results:*** *After 8 weeks of supplementation with L-5-MTHF, the RBC folate concentration increased significantly (283.026±219.578 vs 780.244±221.878) after (vs before) use , with a significant P value of 0.000 (P<0.01).*

***Conclusions:*** *Our findings suggest that L-5-MTHF is able to improve RBC folate significantly in the short term. L-5-MTHF is a viable alternative to improve folate deficiency and is feasible for pregnant females. It can also help them achieve target RBC folate concentration.*

**Keywords:** Folic Acid, L-5-Methyltetrahydrofolate-Calcium

## Abbreviations

- **L-5-MTHF-Ca:** L-5-Methyltetrahydrofolate-calcium.
- **5-MTHF:** 5-Methyltetrahydrofolate
- **FA:** Folic Acid
- **RBC:** Red Blood Cell
- **NTD:** Neural Tube Defect

## Introduction

Folate (also known as vitamin B9) is a water-soluble vitamin essential for DNA synthesis, RNA transcription, methionine synthesis from homocysteine, and other chemical reactions involved in cellular metabolism [1, 2]. In 2015, the WHO established the red blood cell (RBC) folate concentration of 906 nmol/L as the threshold for optimal neural tube defects (NTDs) prevention in populations [3]. Recent studies have found that the RBC folate level of a substantial proportion of maternal women in China is low (<906 nmol/L), and there is a general lack of awareness regarding the importance of adequate folate intake during pregnancy [4]. This deficiency is concerning as it is associated with an increased risk of NTDs and other congenital abnormalities in offspring [5]. It is crucial to implement strategies to improve folate status among pregnant women. Currently, the main method for treating folate deficiency is supplementation with folic acid (FA); however, there is growing controversy over whether to supplement with other forms of folate, such as L-5-methyltetrahydrofolate (L-5-MTHF) [6, 7].

FA is the synthetic, unsubstituted, and oxidized form of folate, which is not typically found in natural food sources. This form of folate has been widely used in dietary supplements and food fortification due to its stability and effectiveness in preventing folate deficiency-related health issues, such as NTDs during pregnancy [8]. Natural food sources of folate primarily contain various reduced forms of the vitamin, such as L-5-MTHF, which

is the active form utilized by the human body [9]. Unlike FA, these natural forms are more readily metabolized and utilized, leading to a lower risk of unmetabolized FA accumulation in the bloodstream [10]. Furthermore, studies have shown that L-5-MTHF supplementation can increase blood folate concentrations more effectively than FA, suggesting that it may be a preferable alternative for some individuals [11]. This raises important questions about the optimal form of folate supplementation, especially in populations at risk for deficiency or those with specific health conditions.

The low RBC folate levels among pregnant women highlight a pressing need for targeted interventions to improve maternal and child health outcomes. In the current study, we explore the effectiveness of active folate in improving RBC folate levels in women preparing for pregnancy. Active folate, particularly in the C-crystalline form of L-5-methyltetrahydrofolate calcium (L-5-MTHF-Ca), has been developed, potentially leading to better outcomes in folate status among women of reproductive age.

## Methods

### Study Subjects

The study population comprised 53 pregnant women (aged 25-40 years), selected from patients with folate deficiency (RBC folate level <906 nmol/L), who visited the Luo Yang Central Hospital Affiliated to Zhengzhou University from November 2023 to September 2024.

### Study Design

This study was a single-center single-arm interventional study. Study subjects were treated with 451μg C-crystalline form of L-5-MTHF-Ca (equivalent to 400μg FA). Each subject took powder containing L-5-MTHF-Ca for 8 weeks, with one sachet a day and informed consent was taken from all participants. Exclusion criteria include the start of other folate supplements within enrollment; Long time to pregnancy (>2 months). Finally, 33 patients were enrolled.

### Laboratory Analysis

Venous blood samples were drawn on admission and on follow-up day in week 8. Blood samples were analyzed for RBC folate with the use of a newly developed liquid chromatography tandem MS (LC-MS/MS) method [12]. The blood sample is centrifuged for 10min at the normal temperature of 2000g, sucking away the blood plasma, adding 4ml of precooled physiological saline, fully shaking up, centrifuging for 10min at 2000g, and sucking away the supernatant. Then, precooled physiological saline of about twice the volume of the erythrocytes was added thereto, and the erythrocytes were suspended by gentle shaking to prepare a physiological saline suspension of erythrocytes (hereinafter referred to as an erythrocyte suspension). 100μl of folate extract (1% ascorbic acid, 1μg/ml GGH recombinase, isotope-labeled 5-MTHF internal standard) was added to 100μl of the erythrocyte suspension and the folate standard solution, respectively, and incubated at 37 ℃ for 1 hour. Then 500μl of 10% trichloroacetic acid (TCA) was added, and the mixture was left to stand for 30 minutes, centrifuged at 13000rpm and 4 ℃ for 10 minutes, and 100μl of the supernatant was taken for folate LC-MS/MS detection. RBC folate concentration = (folate concentration in erythrocyte suspension) / (hematocrit of erythrocyte suspension).

### Statistical Analysis

All data were analyzed using SPSSPRO. The RBC folate levels before and after taking L-5-MTHF-Ca were analyzed using paired t-tests. Statistical significance is indicated by *, where P <0.05, ** where P <0.001, and ***, where P <0.0001.

## Results

### L-5-MTHF-Ca Significantly Increases RBC Folate Levels

Figure 1 illustrates the changes in RBC folate concentration after 8 weeks following the administration of L-5-MTHF-Ca. The pharmacokinetics of L-5-MTHF suggest that it may be more efficiently utilized by the body compared to traditional FA supplements [13]. Pregnant women with folate deficiency who continuously consumed L-5-MTHF-Ca for 8 weeks showed a significant increase in RBC folate concentration. Moreover, the response of RBC folate to L-5-MTHF supplementation appears to be baseline-dependent, with lower baseline resulting in greater increases in folate concentrations over time.

### L-5-MTHF-Ca Supplementation Achieves RBC Folate Levels Given Level Within a Short Period

Women with a starting RBC folate below the population mean (i.e., ≤ 550 nmol/L) have difficulty achieving optimal RBC folate concentrations within 2 months with a FA dose of 400 μg/d. A proportion of females who start supplementation after a positive pregnancy screening test. A model of RBC folate response to FA supplementation has been reported [14]. The prediction equation for post-intervention RBC folate concentration was as follows: RBC folate (nmol/L) after 4 weeks on 400μg/d FA = 25 + 1.27 × baseline RBC folate. The results from the analysis of differences between the measured RBC folate and predicted RBC folate are shown in Table 1. After 8 weeks of L-5-MTHF-Ca supplementation, the RBC folate concentration increased significantly, statistically significant with a p-value of 0.000 ($p < 0.01$), and was also significantly higher than the predicted value, statistically significant with a p-value of 0.003 ($p < 0.01$).

**Table 1: Paired Sample T-test Results of RBC Folate at Baseline and After 8 Weeks and Measured Values and Predicted Values**

| Paired variables | Mean ± SD | | | t | df | P | Cohen's d |
|---|---|---|---|---|---|---|---|
| | Baseline RBC folate, nmol/L | 8 weeks RBC folate, nmol/L | D-value | | | | |
| | 283.026±219.578 | 780.244±221.878 | -497.218±-2.3 | -7.927 | 32 | 0.000*** | 1.38 |
| | Measured concentrations | Predicted from total loading models | | | | | |
| | 780.244±221.878 | 513.243±354.158 | 267.001±-132.28 | 3.22 | 32 | 0.003*** | 0.561 |

**Note: ***, **, and * represent 1%, 5%, and 10% significance levels, respectively.**

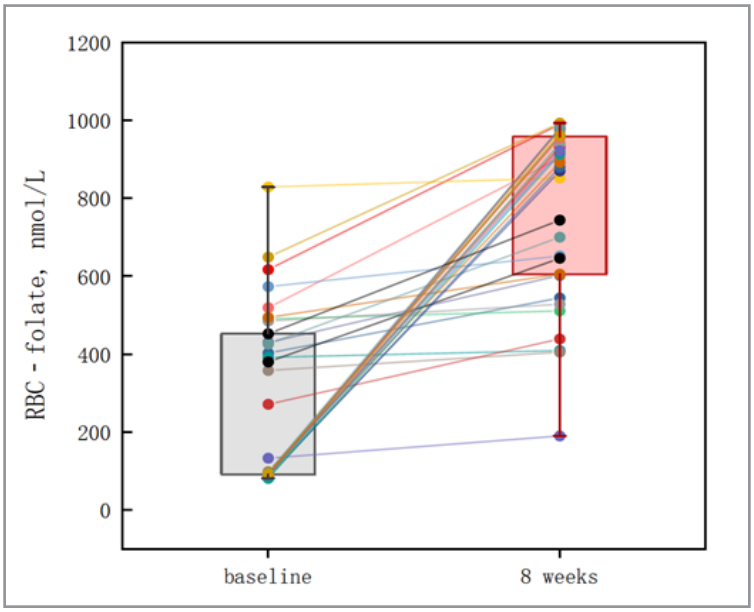


**Figure 1:** Concentrations of RBC Folate Biomarkers Measured in 33 Pregnant Women at Baseline and 8 Weeks, and Their Changes According to Allocation to 451 µg/d L-5-MTHF-Ca

## Discussion

The effect of L-5-MTHF-Ca supplementation in rapidly increasing RBC folate concentrations to the putative target level was shown in our study. Folate supplementation is crucial for various physiological functions, particularly in preventing neural tube defects during pregnancy. A study that modeled RBC folate response to supplementation indicated that individuals with lower baseline RBC folate concentrations may require more time or higher doses of FA to reach optimal levels for health benefits. Therefore, it is essential for women with low RBC folate levels to consider personalized supplementation strategies and possibly longer supplementation durations to ensure they achieve optimal folate levels for reproductive health and overall well-being. Recent studies have highlighted the differences in efficacy between traditional FA supplementation and the use of L-5-MTHF, a more bioactive form of folate [15, 16]. A preliminary evaluation revealed that measured RBC folate concentrations on L-5-MTHF post-intervention were higher than those predicted by published FA supplementation, particularly in women with low initial RBC concentrations.

Folate status in RBCs is a more stable indicator of an individual's folate levels compared to plasma folate, which can fluctuate based on recent dietary intake [17]. This is particularly significant given the importance of maintaining adequate folate levels for various physiological functions, including DNA synthesis and repair, as well as the prevention of neural tube defects during pregnancy. The concentration of folate (primarily 5-MTHF) in the bloodstream is estimated to be around 5-30 nM, whereas the intracellular folate pool in RBCs is considerably higher. Thus, intracellular RBC folate accumulates during erythropoiesis, mature RBCs rarely exchange their folate content with plasma folate and keep their folate content until the end of their lifespan [18]. A study comparing the pharmacokinetics of sodium and calcium salts of L-5-MTHF with FA found that L-5-MTHF resulted in higher peak plasma concentrations and a more favorable area under the curve (AUC) for plasma L-5-MTHF levels. Folate from L-5-MTHF supplements can be loaded into the mature RBCs with low folate concentrations. This indicates that L-5-MTHF may be more effective in raising folate levels in the bloodstream to achieve optimal folate status, which is critical for maintaining adequate folate status in populations at risk of deficiency.

This is a pilot study aiming to report on our preliminary data regarding the effects of L-5-MTHF supplementation. A major limitation of the study is that it was designed as a single-centered single-arm study. A significant proportion of women with folate deficiency reported that their current pregnancy was unplanned, so FA supplementation may not be undertaken. In this context, our study will focus on the feasibility of administering L-5-MTHF, and participant adherence to the supplementation regimen. By analyzing the outcomes from this study, we aim to establish a foundation for future research that could include more extensive trials with control groups to validate our findings.

## Conclusions

Our study aims to acquire initial insights into how active folate supplementation can effectively enhance red blood cell folate levels in women preparing for pregnancy. L-5-MTHF supplementation leads to higher RBC folate concentrations in the short term. In conclusion, while FA remains the standard treatment for folate deficiency, the potential benefits of alternative forms of folate, such as L-5-MTHF, warrant further investigation to determine their efficacy and safety in various populations.

## Availability of Data and Material

All the data in this study were available in the figures in the main text and supplemental documents of this manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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