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SHORT COMMUNICATION

Differential Behavior of Serum and Red Blood Cell Folate During a Treatment with Levofolinic Acid. Gender Differences

ANA M. LENDINEZ [1], ARTURO R. PALOMARES [1, 2], BEATRIZ PEREZ-NEVOT [1], MIRIAM CORTÉS RODRÍGUEZ [1], MAXIMILIANO RUIZ-GALDON [1, 3], ARMANDO REYES-ENGEL [1]

[1] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Málaga, Spain*
[2] *Instituto de Fertilidad Clínicas Rincón, Rincón de la Victoria Málaga, Spain*
[3] *Hospital Clínico Universitario Virgen de la Victoria, Spain*

SUMMARY

Background: **Folates are essential nutrients that maintain nucleotide synthesis and methylation reactions. Folate levels depend essentially on the diet. In the present work, the changes in the folate-homocysteine (Hcy) metabolic axis were studied in response to treatment with levofolinic acid.**
Methods: **49 college students (23 men and 26 women) underwent a treatment voluntarily with 5 mg/day levofolinic acid for one month. Serum and red blood cell folate, vitamin B12, and Hcy levels were determined on days 2, 5, 10, and 30 during treatment and 30 days after completion of treatment.**
Results: **Serum folate and Hcy levels showed a plateau beginning on day 10, while red blood cell folate increased towards treatment completion. Gender differences were found in basal levels of Hcy, these differences remaining until the 10th day of treatment and reappearing 30 days after the treatment was finished. Between gender differences in treatment evolution were found only in percentage changes in red blood cell folate in women and men at day 30 of treatment.**
Conclusions: **There is a compartmentalization of folates in the body that presents a plateau in serum and an erythrocyte reservoir. Folate metabolism presents differential features between genders. The greater physiological need for folate in women of childbearing age could be the determining factor in this difference.**
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KEY WORDS

folate treatment, red blood cell folate, homocysteine, gender difference, hormones

LIST OF ABBREVIATIONS

Hcy - Homocysteine
RBC folate - red blood cell folate

INTRODUCTION

Folates are essential nutrients, and their levels depend on diet and, to a lesser extent, on genetic variants. In one-carbon metabolism (OCM), folates transfer one-carbon fragment involved in nucleotide synthesis, methylation, and metabolism of homocysteine [Hcy] [1].
The proven effect of folate intake for the prevention of neural tube defects during perinatal periods, as well as in the prevention of cardiovascular disease, has led developed countries to take measures for mandatory folate supplementation for certain foods [2]. However, the benefit of the effect of folate overdose is debatable in other processes such as carcinogenesis [3].
The effect of both dietary and pharmacological folate supplementation in OCM has been studied extensively in various populations, including newborns [4], the elderly [5], and young people [6], and a different absorption and catabolism depending on age has been demon-

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strated [7].
In this study, we examined the functioning of the folate-Hcy metabolic axis in response to treatment with levofolinic acid in a group of healthy young people by measuring serum and red blood cell (RBC) folate, vitamin B12, and plasma Hcy over time. We assessed distinct evolution of the compartmental distribution of folates and physiological differences between sexes based on the changes in biochemical indicators.

MATERIALS AND METHODS

Subjects

Forty-nine volunteers, aged 18 to 25 years, were recruited among university students. After obtaining the approval of the University Ethics Committee, we informed all the participants of the objectives of the study and requested their written informed consent to obtain blood samples. Excluded from the study were all subjects with a history of hepatic, renal, gastrointestinal or cardiovascular diseases, as well as those who reported receiving treatment in the three months prior to the study with folate, vitamin B12 and B6, multivitamins, anticonvulsants, antimalarial drugs or oral contraceptives. In compliance with the exclusion criteria, the final group consisted of 49 Caucasian subjects: 23 men and 26 women.

Treatment

Treatment consisted of the oral administration of 5 mg/day of calcium levofolinate (Isovorin®, Pfizer, New York, NY, USA) for 30 days.

Laboratory samples

Fasting blood samples were taken two days before treatment, on days 2, 5, 10, and 30 and 30 days after having finished the treatment. Serum and RBC folate levels, vitamin B12 and Hcy were determined.
Immunonephelometric quantification of Hcy was carried out in EDTA plasma samples using a BNII ® Autoanalyzer (Siemens, Deerfield, IL, USA). Serum samples were used to determine vitamin B12 and serum folate. Red blood cell folate concentrations were measured using EDTA-treated blood after cell lysis was performed at a 1:20 dilution with 1% ascorbic acid. Vitamin B12 and folate values were determined by simultaneous radioimmunoassay (RIA) with solid phase separation without boiling (Solid Phase No Boil Duacont®, DPC, Los Angeles, CA, USA), based on the method of Chen [8]. Two competitive RIAs were performed in the same tube, using two molecules labeled with different isotopes, ^{57}Co vitamin B12 and ^{125}I folic acid.

Statistical analysis

The percentage changes from baseline were used for the statistical analysis in addition to the absolute levels of biochemical parameters. The Student's *t*-test for paired data was performed for comparison of the days. The analysis of the correlation between parameters was performed by calculating the nonparametric Spearman's correlation coefficient, considering the value of r as significant when $p < 0.05$. Statistical analysis of the regression variables was carried out using the least squares method. One-way analysis of variance (ANOVA) was used with the Bonferroni post-test correction to detect differences during treatment between the men and women. All p values were two-tailed and statistical significance was defined as $p < 0.05$.
We used the statistical package GraphPad Prism 6®.

RESULTS

Baseline measurements of serum and RBC folates did not show gender differences, just a significant difference is observed in Hcy (Table 1). During treatment these sex differences in Hcy remained until the 10th day and reappeared 30 days after the treatment was finished (Table 1). No gender differences were observed in serum and RBC folates during treatment. We also studied the correlation of Hcy with serum and RBC folates. The best-fitting regression line was the potential fit ($y = a \cdot b^x$). The largest contribution to the reduction in Hcy was effected by RBC folate (males: $y = 81.46x^{-0.323}$, r = - 0.671, $p < 0.01$; females: $y = 65.86x^{-0.323}$, r = - 0.685, $p < 0.01$).
When analyzing the percentage changes of these parameters in all groups, serum folate and Hcy had more pronounced variations in the first few days of treatment until the 10th day. Between days 10 and 30 the Hcy rate fell more slowly, whereas serum folate rate showed a plateau. However, the percentage of RBC folates increased throughout the whole treatment period (Figure 1). Comparison of the percentage changes between genders in the biochemical parameters measured showed no significant differences in Hcy or serum folate (Figure 2). The only difference found was in the treatment outcome between RBC folate levels reached in women and men at day 30 of treatment, with a higher rate in women (384.22 ± 155.79 vs 485.16 ± 196.81%, $p < 0.001$).
We did not find a significant difference in B12 levels (data not shown).

DISCUSSION

The study population selected was homogenous in age, based on known age-dependent differences in Hcy levels. Although gender differences in basal levels of Hcy were known and widely described [9], unlike other studies, we included earlier measurements, from the start of treatment, to detect the immediacy of the effect of folate intake on Hcy levels. We observed the largest decrease in Hcy on the second day. However, this effect could also be due to the fact that 5-formyltetrahydrofolate (levofolinic acid) is converted mainly into 5-meth-

Table 1. Hcy measurements.

Days of treatment	Males	Females	p
0	11.33 ± 2.88	9.48 ± 3.01	< 0.01
2	8.69 ± 2.16	6.42 ± 1.53	< 0.001
5	7.68 ± 1.29	5.90 ± 1.06	< 0.01
10	6.58 ± 1.12	5.33 ± 0.90	> 0.05
30	6.20 ± 1.08	4.86 ± 0.93	> 0.05
60	8.30 ± 1.51	6.04 ± 1.56	< 0.001

Results presented as mean ± SD µmol/L. p value corresponds to the comparison between sexes by analysis of variance (ANOVA) and Bonferroni post-test correction.

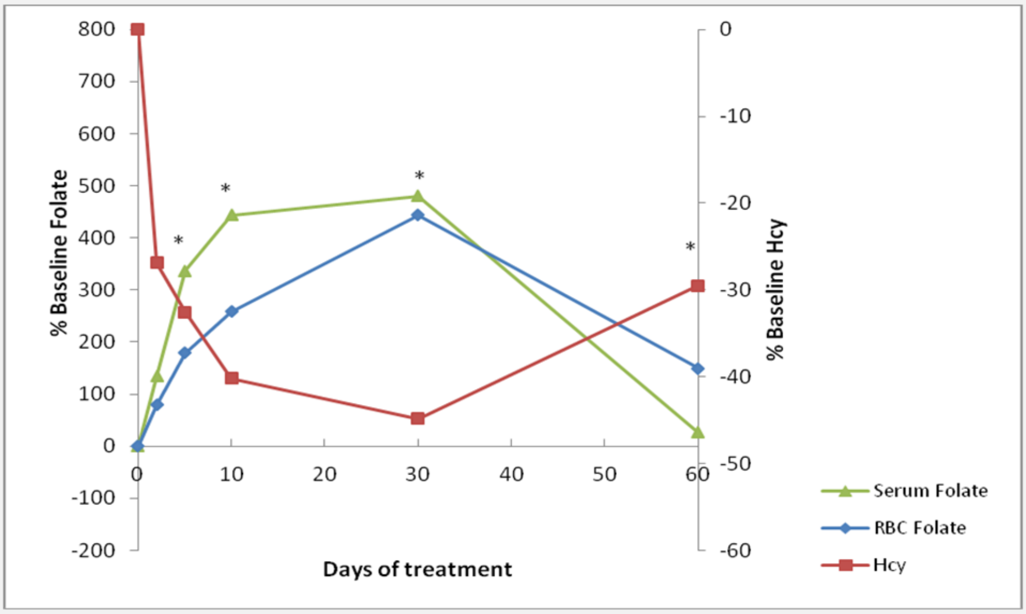

Figure 1. Changes in the biochemical parameters in the population.

Data are presented as mean ± SD of the percentage changes in each parameter from baseline throughout treatment: baseline, days 2, 5, 10, and 30, and 30 days after completion of treatment. * p < 0.01 with Student's *t*-test for paired samples on each day compared to day 2. (RBC Folate - Red Blood Cell Folate, Hcy - Homocysteine).

yltetrahydrofolate, unlike folic acid [10].
The changes in serum and RBC folate presented different patterns: serum folate reached a plateau on the tenth day of treatment whereas RBC folate continued to increase throughout the treatment. A similar effect was detected in the first study performed on folate kinetics [11]. The serum folate plateau always occurs in a dose-dependent manner [12], although at different times during treatment. We found that women reached serum the folate plateau at the 10th day, while men seemed to reach it later. However, these differences were not statistically significant. There appears to be a primary regulation of serum folate levels modulated by the folate reservoir role played by erythrocytes and their renal excretion rate. In the literature, this store of erythrocytes is accepted as an indicator of tissue stores [13], although we have found no studies confirming this, as there are no reviews of folate levels in different cell types. In

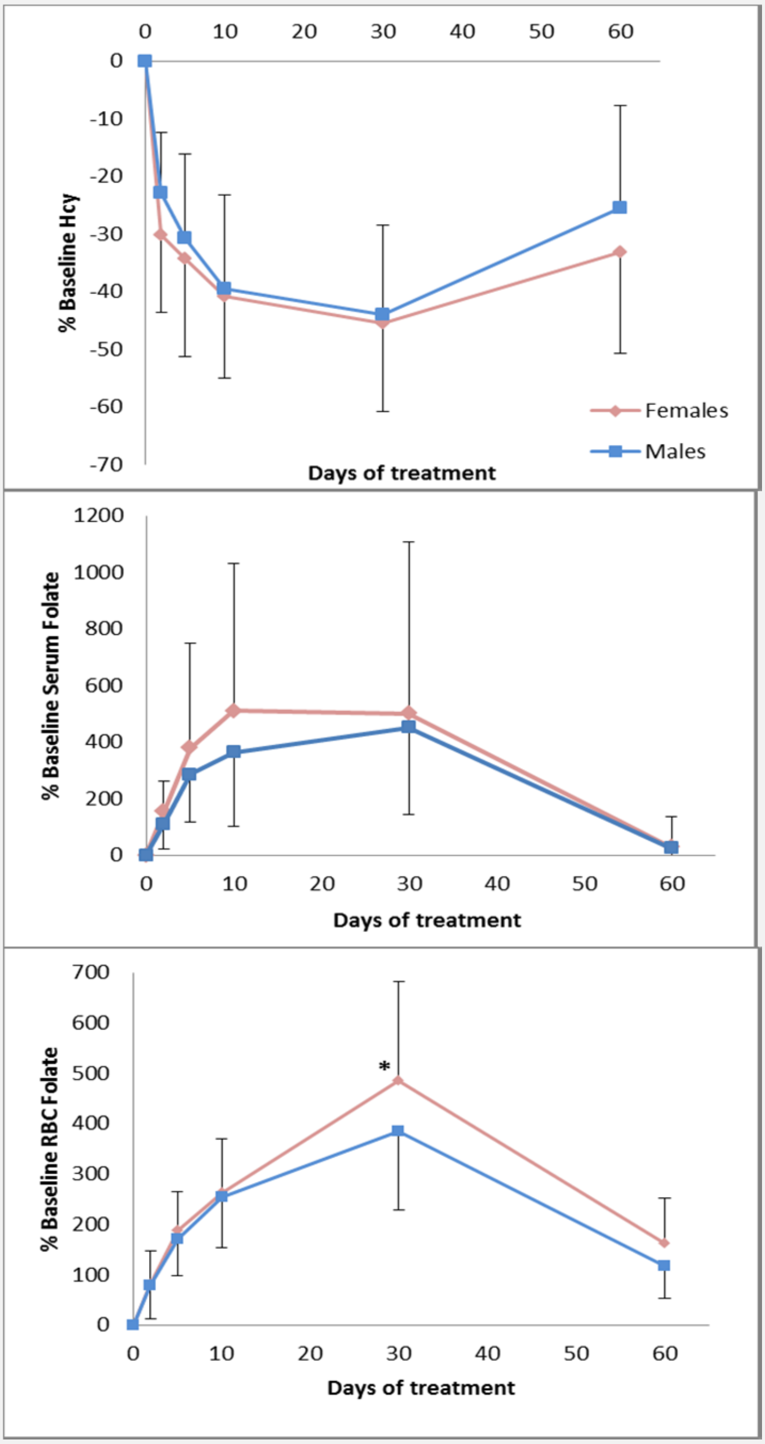

Figure 2. Biochemical parameters by gender.

Results presented as mean ± SD of percentage change from baseline throughout treatment: baseline, days 2, 5, 10, and 30, and 30 days after completion. * p < 0.001; p value corresponds to the comparison between sexes by analysis of variance (ANOVA) and Bonferroni post-test correction.

fact, experts are inclined to use RBC folate as a biomarker of folate status, but acknowledge having "incomplete knowledge" as to whether it is preferable compared to serum levels [14]. It would appear that there is a threshold or maximum concentration of serum folate that cannot be exceeded despite high external input.

Our experimental findings are consistent with the recent publication of a mathematical model to simulate the relationship between plasma levels of folate and metabolites in the methionine cycle [15]. From the two folate parameters, RBC folate correlates best with Hcy, which seems consistent with the fact that these levels of RBC folate most resemble intracellular folate in comparison with serum folate.

Unlike other studies on folinic acid supplementation (50 - 800 µg) in men and women [16], we used a high dose (5 mg) and analyzed the variations, not the concentrations [9]. With this dose we aimed for a potential metabolic saturation effect. The results suggest that we managed to achieve this, given the serum folate and Hcy plateaus found, although not with RBC folate. With this saturation we can rule out the dietary factor as influential in gender differences, leaving hormone status as the main factor that conditions cycle functioning. Regarding hormone regulation of the folate-Hcy cycle, references exist on the role of estradiol [17]. In fact, during the menstrual cycle, differences in Hcy levels have been observed, which are greater in the follicular phase when there is less estrogen [18]. Plasma Hcy equalizes in men and women when women reach menopause, but with hormone replacement therapy the differences are restored [19]. Given the design of our study, the homogeneity in age and the high doses of folate, we can suppose that estradiol marks the differences between genders. *In vitro* studies in tumors have shown the effect of estrogen on the expression of folate receptors/transporters [20,21], suggesting that this could be the mechanism by which men metabolize folates differently and women accumulate more folate and present lower baseline Hcy than men.

Although studies on treatments with folates and their effects are abundant, to date, our study offers a comprehensive analysis of their compartmentalization and physiological tissue distribution. In this work, we found a compartmentalization of folate in the body that has a threshold in serum but an ample erythrocyte reservoir that is difficult to saturate. Folate metabolism presents differential characteristics between sexes, with women having a greater RBC folate storage capacity, possibly through a quantitatively greater transport system and mediated by estrogen levels. The greater physiological need for folate in women of childbearing age could be the determining factor in this difference.

Authors' Contributions:

AML, ARP, BPN, and MCR contributed to the acquisition, analysis, and interpretation of data and drafted the manuscript. AML and MRG performed the statistical analyses. MRG also participated in the design and coordination of the study and helped to draft the manuscript. ARE conceived and designed the study, oversaw the analysis, and contributed to the interpretation of the results and preparation of the final manuscript. All authors have read and approved the final manuscript.

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Declaration of Interest:

The authors have declared there are no competing interests.

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Correspondence:
Ana M. Lendinez
Department of Biochemistry and Molecular Biology
Faculty of Medicine
University of Málaga
Boulevard Louis Pasteur s/n. 29071
Malaga, Spain
Tel.: +34 952131533
Fax: +34 952131534
Email: analendinezramirez@gmail.com