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Abstract

Background: Lack of folic acid intake or genetic abnormalities in folic acid metabolism was correlates with elevated plasma or serum homocysteine concentrations. This case-control analytical study aims to determine the correlation between folic acid and homocysteine levels in severe pre-eclampsia and normal pregnancy. **Methods:** We enrolled 46 pregnant women (age 20─35 years) with severe pre-eclampsia or normal pregnancy at a government hospital in Padang, Indonesia, between March and May 2015. The samples size was selected by consecutive sampling. Then, we determined folic acid and homocysteine levels using ELISA and statistical analysis using the independent *t*-test and Pearson correlation. **Results:** We observed a difference in folic acid levels between severe pre-eclampsia (39.48 ± 9.40 ng/mL) and normal pregnancy (47.04 ± 13.20 ng/mL, $p < 0.05$). A difference was also observed in homocysteine levels between pre-eclampsia (18.52 ± 0.41 pmol/mL) and normal pregnancy (17.80 ± 0.73 pmol/mL, $p < 0.05$). The correlation between folic acid and homocysteine in severe pre-eclampsia and normal pregnancy was negative ($r = -0.034$, and $r = -0.222$, $p > 0.05$, respectively). **Conclusions:** Low folic acid levels tend to increase homocysteine levels in severe pre-eclampsia, whereas high folic acid levels tend to lower homocysteine levels in normal pregnancy.

Keywords: folic acid, homocysteine, pre-eclampsia, pregnancy

Introduction

To date the cause of pre-eclampsia remains unclear. However, endothelial cell activation has been the centre of contemporary understanding of the pathogenesis of pre-eclampsia over the past two decades.[1] Healthy endothelial cells can maintain vascular integrity, preventing platelet adhesion and affecting the vascular smooth muscle tone. The presence of endothelial damage results in an inability to maintain the function resulting in the increased vascular permeability, platelet thrombosis, and increased vascular tone.[1] Vascular damage to poor maternal uteroplacental circulation and foetal umbilical accounts for pre-eclampsia. Reportedly, one of the factors causing vascular damage to pre-eclampsia is homocysteine.[2] In facts, several have reported that homocysteine is elevated in women with severe pre-eclampsia.[3-5]

Homocysteine is a non-proteinogenic α-amino acid and an intermediate compound produced in the methionine metabolism. Methionine is an essential amino acid containing sulphur obtained from the intake of protein-rich food.[6] Reportedly, elevated total plasma homocysteine levels are a risk factor for vascular disease.[7]

Hyperhomocysteinemia causes endothelial dysfunction through several direct toxic mechanisms and oxidative stress.[8,9] In addition, hyperhomocysteinemia decreases the bioactivity of nitric oxide (NO), a relaxing smooth muscle factor that is a vasodilator and lubricant to prevent the attachment of low-density lipoprotein and blood cells, as well as in response to various stimuli.[6] Moreover, NO induces vasodilation and regulates vascular resistance. The NO reduction causes endothelial dysfunction Reportedly, the disruption of endothelial function as a vasodilator plays a role in the pathophysiology of hypertension.[8]

Primarily, homocysteine metabolism depends on three enzymes (methionine synthase, 5,10-methylenetetra-hydrofolate reductase-, and cystathionine β- synthase (CBS) and some cofactor vitamins (B6, B12-, and folic acid). Both folic acid and vitamin B play a vital role in various biochemical processes, including homocysteine metabolism.[9] In fact folic essential in two biochemical cycles involved in the biosynthesis of the deoxy-ribonucleic acid (DNA) and one-carbon metabolism (DNA, lipid and protein methylation). In addition, folic acid is a major substrate for the conversion of homocysteine to methionine.[6] Studies have established

that a lack of folic acid intake or genetic abnormalities in folic acid metabolism is correlates with elevated plasma or serum homocysteine concentrations.[3,6,10–12] Furthermore, low folate levels have been detected in patients with pre-eclampsia.[13] A correlation between folate deficiency and an increase in total homocysteine (tHcy) has been extensively reported.[2,14–16] However, some studies have reported no correlation between folic acid and homocysteine levels.[3,8,12,17] The controversy surrounding the role of folic acid in increasing homocysteine levels prompted the researchers to seek further clarification. Hence, this study aims to determine the correlation between folic acid level and plasma homocysteine in pre-eclampsia and normal pregnancy.

Methods

Study design and study cohort. This case-control analytical study was conducted at a government hospital in Padang, Indonesia, from March to May 2015. We enrolled all normal pregnant women with 37–40 weeks gestation and severe pre-eclampsia with 20–40 weeks gestation. The study cohort comprised pregnant women with severe pre-eclampsia or those with normal pregnancy who fulfilled the inclusion and exclusion criteria. Severe pre-eclampsia was a condition of hypertension in a pregnancy diagnosed after 20 weeks' gestation with a blood pressure $\geq$ 160/110 mmHg and accompanied by proteinuria $\geq$ +1 with dipstick examination. The inclusion criteria were as follows: single and double pregnancy, nulliparity and multiparity and age 20–35 years. Conversely, the exclusion criteria were as follows: patients undergoing treatment that affected homocysteine levels (epilepsy, cancer and asthma), patients consuming beer or the like containing 1 cup alcohol/day, drinking >5 cups of coffee/day and smoking >1 cigarette/day; hystory of heart disease, kidney disease, hypertension, diabetes mellitus, asthma and epilepsy based on anamnesis and medical record.

This study protocol received ethical approval from Research Ethics Committee of Faculty of Medicine Andalas University, Padang (No. 024/KEP/FK/2015). Participants were given an explanation before approval and then signed an informed concent. The sample size was determined based on the formula for calculating the sample size using two independent populations[18] as 46 (each group included 23 individuals). Sampling was performed using consecutive sampling technique.

Data collection. We assessed folic acid and homocysteine levels using spectrophotometer with the ELISA method, conducted at Biomedical Laboratory Faculty of Medicine Andalas University. Using the Human VB9/ Folic Acid ELISA kit (Elabscience), we detected folic acid levels (0–200 ng/mL). In addition, we used the Human Homocysteine ELISA Kit (Elabscience) to detect homocysteine levels (0–60 pmol/mL). From all

participants, we obtained 3 cm^3 of venous blood plasma from the antecubital vein area in the supine position. Then, within 30 min of drawing blood, the blood sample was stored in an EDTA-containing tube, carefully shaken and centrifuged at 1000 $\times g$ for 15 min at 2 °C–8 °C. Next, we removed the centrifuged plasma into the sample cup. Finally, the blood samples were sent to the Biomedical Laboratory of the Faculty of Medicine Andalas University and stored at –80 °C until examination.

Statistical analysis. Samples were first analysed in the laboratory and then processing and analyzing data used IBM SPSS Statistics Version 20, with 95% confidence intervals ($\alpha = 0.05$; $\beta = 80\%$). *P* value was used to analyse significance criteria; if $p \leq 0.05$ means significant. Normality test was analysed by One-Sample Kolmogorov Smirnov. The numerical data with normal distribution was calculated on mean and standard deviations. The categorical data were calculated in frequency and analysed by Chi-square The difference between folic acid and homocysteine was analysed by independent *t*-test. Pearson correlation test was used to analyse correlation between folic acid and homocysteine.

Results

We examined 46 pregnant women in this study. In the severe pre-eclampsia group, the mean age of pregnant women (29.65 ± 4.30 years) tended to be older than that in the normal pregnancy group (28.83 ± 3.60 years); we observed no difference between the two groups ($p = 0.484$). The mean gestational age in the severe pre-eclampsia group (35.17 ± 3.48 weeks) was shorter compared with the normal pregnancy group (38.30 ± 1.14 weeks); we observed a marginal difference between severe pre-eclampsia and normal pregnancy ($p = 0,000$).

The mean body mass index before pregnancy in the severe pre-eclampsia group (22.60 ± 4.32 kg/m^2) was lower than normal pregnancy (23.40 ± 5.09 kg/m^2); no difference was observed between the two groups ($p = 0.564$). Based on parity, we determined that eight women (34.8%) had severe pre-eclampsia, whereas, amongst multiparas, 15 women (65.2%) had severe pre-eclampsia: we observed no assosiation parity with severe pre-eclampsia ($p = 0.75$). Based on the history of pre-eclampsia, three women (20%) having mothers with a history of pre-eclampsia had severe pre-eclampsia in this pregnancy; no association PE history with severe pre-eclampsia; $p = 0.18$ (Table 1).

The mean folic acid levels of severe pre-eclampsia (39.48 ± 9.40 ng/mL) tended to be lower than that in normal pregnancy group (47.04 ± 13.20 ng/mL); we observed difference between severe pre-eclampsia and normal pregnancy ($p = 0.031$; $p < 0.05$). The mean homocysteine levels of severe pre-eclampsia (18.52 ± 0.41

Table 1. Characteristics of the Study Cohort

Variable	Severe pre-eclampsia ($n = 23$) (mean ± SD)	Normal pregnancy ($n = 23$) (mean ± SD)	p
Age of mother (year)	29.65 ± 4.30	28.83 ± 3.60	0.484[#]
Duration of pregnancy (weeks)	35.17 ± 3.48	38.30 ± 1.14	0.000[#**]
BMI before pregnancy (kg/m²)	22.60 ± 4.32	23.40 ± 5.09	0.564[#]
Parity			
- Nulliparity	8 (34.8)	6 (26.1)	0.75*
- Multiparity	15 (65.2)	17 (73.9)	
PE History			
- Yes	3 (20%)	0 (0%)	0.18*
- No	12 (80%)	17 (100%)	
Folic acid (ng/mL)	39.48 ± 9.40	47.04 ± 13.20	0.031[#**]
Homocysteine levels (pmol/mL)	18.52 ± 0.41	17.80 ± 0.73	0.000[#**]

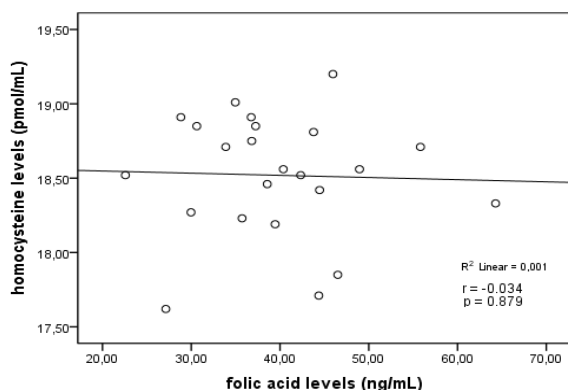[#]independent *t*-test **p* < 0.05
**Chi-square test

Figure 1. **Correlation Between Folic Acid Level and Homocysteine in Severe Pre-Eclampsia**
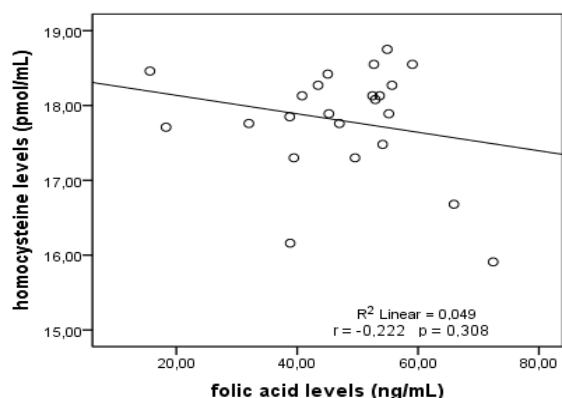

Figure 2. **Correlation Between Folic Acid Level and Homocysteine in Normal Pregnancy**

pmol/mL) tended to be higher than that in normal pregnancy (17.80 ± 0.73 pmol/mL); we observed marginal difference between the two groups ($p = 0,000$).

In the severe pre-eclampsia group, the correlation was insignificant between folic acid and homocysteine ($r = -0.034$; $R^2 = 0.001$; $p = 0.879$; Figure 1). In addition, the correlation between folic acid and homocysteine in normal pregnancy was $r = -0.222$ ($R^2 = 0.049$), suggesting insignificant negative correlation between folic acid and homocysteine in normal pregnancy ($p = 0.308$; Figure 2).

Discussion

Correlation between folic acid levels and homocysteine in pre-eclampsia. Hyperhomocysteinemia is one of the leading factors that causes vascular damage to pre-eclampsia.[2] Most studies have reported that homocysteine levels are elevated in women with severe pre-eclampsia.[3–5] Homocysteine causes oxidative stress accounting for endothelial cell damage that plays a role in the patho-physiology of pre-eclampsia. Homocysteine can cause direct vascular damage and may increase the reactive oxygen species (ROS) production in endothelial and smooth muscle cells.[11] In vitro studies have reported homocysteine to cause endothelial cell changes. Reportedly, elevated homocysteine levels alter the function of the vascular endothelial cell surface from anticoagulant to pro-coagulant and smooth muscle cell proliferation.[19]

Homocysteine rapidly reacts with NO to form S-nitroso-Hcy, which acts as a potent antiplatelet agent and vasodilator; this formation might decline the peroxide production from homocysteine.[19] Vascular injury is attributed to an imbalance of the NO production of dysfunctional endothelial cells and homocysteine concentrations. In addition, homocysteine reduces the expression of antioxidant enzyme glutathione peroxidase and enhances the natural killer factor B.[11]

Homocysteine reduces cell viability in a time and concentration dependent manner.[19] In a study, the

highest cytotoxicity was reported by 80-μM homocysteine resulting in 80% of cell death after 5 days. Furthermore, the study reported a marked decline in the cell viability of 35% after 5-day incubation with 40-μM homocysteine.[19]

One of the functions of vitamins is enzyme cofactors. Vitamin B6, B12 and folic acid are vital cofactors for the methionine–homocysteine metabolism. Thus, low availability of vitamin B (B6, B12 and folic acid) prevents remethylation of homocysteine into methionine, thereby accumulating homocysteine.[19] Vitamin B decreases homocysteine without improving endothelial dysfunction or hypercoagulability. Recent findings have suggested that homocysteine accumulates secondary to immune activation–related increased oxidative stress. The correlation between cardiovascular disease and homocysteine could stem from vitamin B deficiency or only the reactivity of vascular changes when folate levels are low simultaneously.[19] Furthermore, some vitamin therapies prevent vascular complications of homocystinuria, and vitamin use is related to a lower risk of vascular disease in the general population.[20,21] However, Previous studies reported a correlation between low plasma folate levels and high homocysteine concentrations in pre-eclampsia.[2,16,22,23] Folate acts as a precursor for 5-methyltetrahydrofolate, methyl donor for Hcy remethylation to methionine,[24] thus, Hcy auto-oxidation produces oxidised disulphide, two protons (H^+) and two electrons (e^-) that stimulate the formation of the ROS does not occur.[24,25]

In this study, the absence of a correlation between folic acid levels and homocysteine attributed to higher folic acid levels than normal. High folate intake or supplementation before 20 weeks of pregnancy assumed to play a role in decreasing homocysteine levels. In addition, this study establishes that folic acid may not be a major predictor of elevated homocysteine levels in pre-eclampsia. There is still much more to be learnt about the relationship between pre-eclampsia and hyperhomocysteinemia. Notably, trans-sulphuration insufficiency (via the CBS mutation or vitamin B6 deficiency) or restriction on remethylation (vitamin B12 deficiency or polymorphic genetic disorder methylenetetrahydrofolate reductase/MTHFR C677T) results in homocysteine accumulation.[25] Reportedly, the thermoplastic MTHFR C677T polymorphic gene reduces 50% enzymatic activity in patients with folate deficiency.[25]

Correlation between folic acid levels and homocysteine in normal pregnancy. In this study, high folic acid levels in normal pregnant women inversely correlated with low homocysteine levels or tended to exhibit lower homocysteine levels. High folate levels could be attributed to folate supplementation during pregnancy obtained during pregnancy screening visits. In addition, it is influenced by folate levels in previous pregnancies that have reached the normal limit or parity distance of

>2 years so that mothers already have high folate reserves for subsequent pregnancies. Some studies reported a correlation between folate supplementation and low homocysteine levels;[14,26,27] however, other studies failed to validate the correlation.[12,28] In addition, some studies reported that doses of 0.65–10 mg/day of folic acid alone or together with vitamin B12 and B6 reduce fasting and post-methionine load levels by 25%–50%, both in healthy subjects and hyperhomocysteinemia and patients with vascular disorders.[20] Usually, the total folate intake of foods and supplements <200–250 μg/day correlates with hyperhomocysteinemia.[20] The intake of 400 μg/day in the second and third trimesters elevates the concentration of red cell folate and umbilical cord folate and prevents reduced serum folate and decreased homocysteine concentrations in pregnancy.[24] Reportedly, higher doses were required to decline the maximal tHcy in groups with impaired renal function or low folate status.[20] Furthermore, plasma homocysteine responded to the folic acid supplementation at a dose of 500–600 μg/day.[29]

Although the highest safe limit for the folate intake remains unclear, it is typically considered up to 1 mg/day for adults; likewise, no consensus exists about the highest safe concentration of blood folate. Serum folate concentrations of >45 nmol/L are considered supraphysiological.[30] In fact, high folate levels have been reported to disguise the symptoms of vitamin B12 deficiency.[20,30] The homocysteine concentration is determined by nutritional and genetic factors.[30] Folate is the most potent homocysteine predictor[15,31] and serves to provide the methyl group for the conversion of homocysteine to methionine, which joins in proteins and other crucial functions related methylation reactions.[30] The most significant genetic predictor is the 677C→T on genes coding MTHFR. Reportedly, individuals with polymorphic homozygotes decreased the MTHFR activity, resulting in the increased homocysteine.[15]

Obwegeser *et al.* reported that hormonal factors affect homocysteine levels during pregnancy.[31] Concentrations of tHcy during pregnancy are induced by hormones and exert a stronger effect than vitamins in lowering homocysteine concentrations.[28] Cortisol and oestrogens cause increased activity of the Hcy–methyltransferase enzyme in the liver and methionine synthase in the kidneys resulting in the increased remethylation from Hcy to methionine.[31] Studies in adult rats administered with cortisol or oestradiol or a combination of both reported a marked decline in plasma homocysteine levels. An experiment concluded that cortisol was more effective in decreasing plasma homocysteine than oestradiol.[31] Another study reported that tHcy concentrations were 29%–60% lower in pregnant women than non-pregnant and reached the lowest levels in the second trimester of pregnancy[28] but increased slowly at the end of the third trimester of pregnancy to attain the

similar concentration as non-pregnant women.[26] Reportedly, increased homocysteine at the end of pregnancy is a natural phenomenon that prepares the uterus for labour by stimulating uterine cell contraction.[26] Moreover, studies on a term pregnancy in pigs reported a myo-metrium spontaneous contraction after induced by homocysteine.[32] In this study, we suspected a mild increment in homocysteine levels to stimulate uterine contractions; however, further research is warranted validate it.

In contrast to Wallace *et al*.,[15] some previous studies[15,21,27] that indicated a correlation between folic acid levels and homocysteine in normal pregnancy. A lack of correlation in this study was attributed to a mild increase in homocysteine levels at term pregnancy. Although this study determined mild hyperhomocys-teinemia in normal pregnancy, it did not cause endothelial cell damage as pre-eclampsia. Assumedly, higher levels of folate (because of intake and supplementation) are observed in normal pregnancy respondents so that high folate levels could maintain endothelial cells. Furthermore, high folate levels prevent free radicals that are formed by auto-oxidation of homocysteine.[24,25]

Limitations. This study has some limitation. First, this study did not review the daily intake of folic acid and protein before. Reportedly, a dietary history of high folic acid increased serum/plasma levels of folate.[27] Plasma or folate serum describes the folate intake in the last few days. A study reported that the diet containing excessive methionine increases homocysteine levels.[33] Second, we also did not assess participants' adherence to folate supplementation during pregnancy and the amount consumed. Folate supplementation increased folate levels during labour.[15] In this study, no folate and homocysteine levels were known before pregnancy or in the first trimester of pregnancy. Besides, blood sampling was performed in a non-fasting state because of the unlikely condition. Furthermore, the difference in the gestational age in both study groups was so significant that it affected the levels of folic acid and homocysteine.

Conclusions

This study reveals an insignificant negative correlation between folic acid levels and homocysteine in pre-eclampsia, as well as in normal pregnancy. Hence, further research is warranted in the MTHFR 677C and larger studies that explore other potential causes.

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Conflict of Interest Statement

All authors declare no conflict of interest.

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