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Folic acid supplementation attenuates hyperhomocysteinemia-induced preeclampsia-like symptoms in rats*☆

Jun Wang[1], Yan Cui[2], Jing Ge[1], Meijing Ma[3]

1 *Department of Gynaecology and Obstetrics, the 202 Hospital of People's Liberation Army, Shenyang 110003, Liaoning Province, China*
2 *Department of Emergency Medicine, General Hospital of Shenyang Military Region, Shenyang 110016, Liaoning Province, China*
3 *Department of Gynaecology and Obstetrics, the Second People's Hospital of Tongliao, Tongliao 280000, Inner Mongolia Autonomous Region, China*

Abstract
Folic acid participates in the metabolism of homocysteine and lowers plasma homocysteine levels directly or indirectly. To establish a hyperhomocysteinemic pregnant rat model, 2 mL of DL-homocysteine was administered daily by intraperitoneal injection at a dose of 200 mg/kg from day 10 to day 19 of gestation. Folic acid was administered by intragastric administration at a dose of 20 mg/kg during the period of preeclampsia induction. Results showed that systolic blood pressure, proteinuria/creatinine ratio, and plasma homocysteine levels in the hyperhomocysteinemic pregnant rats increased significantly, and that body weight and brain weight of rat pups significantly decreased. Folic acid supplementation markedly reversed the above-mentioned abnormal changes of hyperhomocysteinemic pregnant rats and rat pups. These findings suggest that folic acid can alleviate the symptoms of hyperhomocysteinemia- induced preeclampsia in pregnant rats without influencing brain development of rat pups.

Key Words
folic acid; preeclampsia; hyperhomocysteinemia; proteinuria; creatinine; preeclampsia-like symptom; pregnant rats; offspring; brain; nervous system; regeneration; neural regeneration

Research Highlights
(1) A preeclampsia rat model was established using intraperitoneal injection of DL-homocysteine to mimic the manifestation of hypertension, proteinuria and restricted fetal development during pregnancy.
(2) Folic acid supplementation alleviated the symptoms of hyperhomocysteinemia-induced preeclampsia in pregnant rats and folic acid did not produce an obvious influence on the brain development of rat pups.

Jun Wang☆, M.D., Associate chief physician, Master's supervisor, the 202 Hospital of People's Liberation Army, Shenyang 110003, Liaoning Province, China

Jun Wang and Yan Cui contributed equally to this work.

Corresponding author: Jun Wang, the 202 Hospital of People's Liberation Army, Shenyang 110003, Liaoning Province, China
wj202fck@163.com

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INTRODUCTION

Preeclampsia is defined by the onset of hypertension and proteinuria at or after 20 weeks of gestation and is associated with intrauterine growth restriction of the fetus. It is a leading cause of maternal and neonatal mortality and may also increase the risks of cardiovascular disease and diabetes in the offspring of the affected mothers[1-2]. Preeclampsia is likely a two-stage disorder: at stage I (most likely at the late first trimester or early second trimester), a decreased placental perfusion, secondary to abnormal placental developments, develops; and at stage II (most likely at the early third trimester), the maternal syndrome of preeclampsia, secondary to systemic endothelial dysfunction, develops[3]. The etiology of preeclampsia is unknown. A previous study showed that factors produced by the poorly perfused placenta may enter the systemic circulation, activate coagulation and reduce vascular integrity, resulting in the pathophysiologic changes of preeclampsia[3]. However, which factors are responsible for the development of preeclampsia and how they interact with maternal predisposing factors to induce the clinical syndrome of preeclampsia remain elusive[3]. Strong evidence suggests that endothelial cell dysfunction during pregnancy contributes to the development of preeclampsia[4]. The role of homocysteine in vascular endothelial dysfunction has been studied extensively[5].

Homocysteine is toxic to the vascular endothelium and impairs endothelial function by inhibiting the synthesis of endothelium derived relaxing factor, nitric oxide or by increasing its degradation *via* the generation of oxygen-derived radicals such as superoxide radical, peroxynitrite and hydrogen peroxide[6-7]. Hyperhomocysteinemia is a causative agent for systolic hypertension[8] and is associated with preeclampsia[9-11]. Clinical research on hyperhomocysteinemia is difficult, as the onset of the disease is sudden and requires immediate medical assistance to help prevent negative maternal and fetal outcomes. Therefore, animal models have greatly contributed to the advancement of knowledge in this field. However, to date, the disease develops spontaneously in very few rodent models[12]. Although other models have been proposed by administering compounds[13] or performing surgery[14] at mid-term during gestation, they may not mimic the disease appropriately, because it has been shown that the mechanisms involved in preeclampsia occur even before the onset of symptoms[15]. Hence, establishment of animal models would be very useful to elucidate the mechanisms involved in this still poorly characterized disease.

Recent studies have found that supplementation of multivitamins containing folic acid are associated with a reduced risk of preeclampsia[16]. Folic acid may reduce the risk of preeclampsia by improving placental and systemic endothelial function and directly or indirectly by lowering plasma homocysteine levels[17-18]. The purpose of this study was to determine whether the supplementation of folic acid is useful for the prevention of preeclampsia *in vivo*.

RESULTS

Quantitative analysis of animals

Thirty pregnant Wistar rats were randomly and evenly divided into pregnant control, pregnant + homocysteine, and pregnant + homocysteine + folic acid groups. Twenty nonpregnant Wistar rats were randomly and evenly divided into nonpregnant control and nonpregnant + homocysteine groups. In the pregnant + homocysteine and nonpregnant + homocysteine groups, rats received a solution of DL-homocysteine in drinking water. In the pregnant control and nonpregnant control groups, DL-homocysteine was not added to the drinking water. In the nonpregnant + homocysteine and pregnant + homocysteine + folic acid groups, rats which had spurious pregnancy or were mistaken in the grouping were rejected during result analysis. Ten rats from the pregnant control, nonpregnant control, and pregnant + homocysteine groups, and nine rats from the nonpregnant + homocysteine and pregnant + homocysteine + folic acid groups were included in the final analysis.

Effect of folic acid on systolic blood pressure in rats with hyperhomocysteinemia

Systolic blood pressure gradually increased in rats from day 0 to day 18 of gestation. On day 18 of gestation, systolic blood pressure was significantly increased in pregnant rats treated with homocysteine than that in pregnant rats without hyperhomocysteinemia (P = 0.008) (Figure 1). On day 18 of gestation, increased systolic blood pressure was also detected in nonpregnant rats treated with homocysteine compared with nonpregnant controls (P = 0.017). However, on day 18 of gestation, systolic blood pressure significantly decreased in pregnant rats treated with homocysteine and folic acid compared with pregnant rats treated only with homocysteine (P = 0.015).

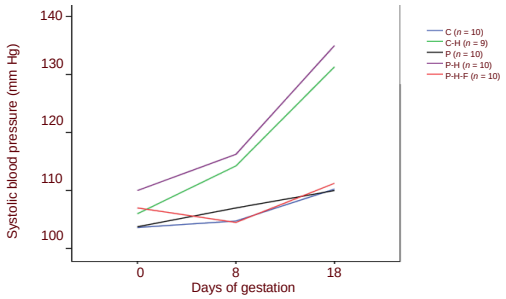

Figure 1 Effect of folic acid on systolic blood pressure in pregnant rats with hyperhomocysteinemia.

On day 18 of gestation, P-H group *vs.* P group ($P = 0.008$); C-H group *vs.* C group ($P = 0.017$); P-H-F group *vs.* P-H group ($P = 0.015$). Data are expressed as mean ± SD. The differences among groups were compared by analysis of variance and *post-hoc* least significant difference test.

C: Nonpregnant control; C-H: nonpregnant + homocysteine group; P: pregnant control; P-H: pregnant + homocysteine; P-H-F: pregnant + homocysteine + folic acid. 1 mm Hg = 0.133 kPa.

Effect of folic acid on urinary protein/creatinine ratio in rats with hyperhomocysteinemia

There was an approximately two-fold increase in urinary protein/creatinine ratio in the pregnant + homocysteine group when compared with the pregnant control group ($P = 0.001$). Importantly, the urinary protein/creatinine ratio did not vary significantly in the nonpregnant + homocysteine group when compared with nonpregnant control group. The urinary protein/creatinine ratio significantly decreased in the pregnant + homocysteine + folic acid group compared with the pregnant + homocysteine group ($P = 0.020$) (Figure 2).

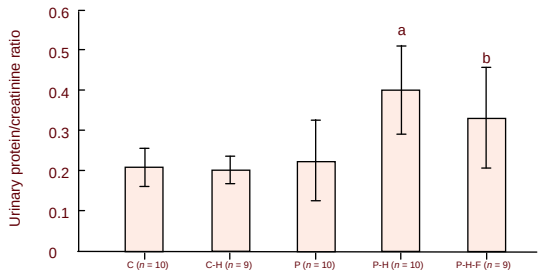

Figure 2 Effect of folic acid on urinary protein/creatinine ratio in pregnant rats with hyperhomocysteinemia.

The results are expressed as protein/creatinine ratio in mg/mL (mean ± SD). $^{a}P = 0.001$, *vs.* P group; $^{b}P = 0.020$, *vs.* P-H group. The differences among groups were compared by analysis of variance and *post-hoc* least significant difference test.

C: Nonpregnant control; C-H: nonpregnant + homocysteine group; P: pregnant control; P-H: pregnant + homocysteine; P-H-F: pregnant + homocysteine + folic acid.

Effect of folic acid on plasma homocysteine levels in rats with hyperhomocysteinemia

Plasma homocysteine levels in the pregnant and nonpregnant rats increased significantly following treatment with DL-homocysteine ($P < 0.001$). Folic acid treatment decreased plasma homocysteine levels in pregnant rats treated with DL-homocysteine ($P < 0.01$) (Figure 3).

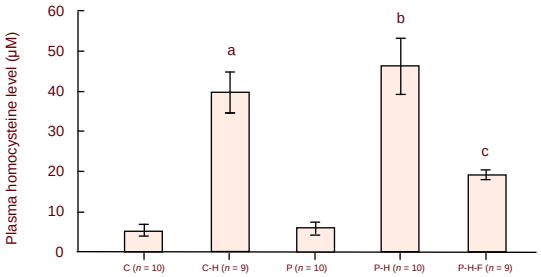

Figure 3 Effect of folic acid on plasma homocysteine levels in pregnant rats with hyperhomocysteinemia.

Data are shown as mean ± SD. $^{a}P < 0.001$, *vs.* C group, $^{b}P < 0.001$, *vs.* P group, $^{c}P < 0.001$, *vs.* P-H group. The differences among groups were compared by analysis of variance and *post-hoc* least significant difference test.

C: Nonpregnant control; C-H: nonpregnant + homocysteine group; P: pregnant control; P-H: pregnant + homocysteine; P-H-F: pregnant + homocysteine + folic acid.

Effect of folic acid on body weight and brain weight of rat pups

Average pup weight and brain weight in the pregnant + homocysteine group were significantly decreased than in the pregnant control group ($P < 0.05$). These values were decreased in the pregnant + homocysteine + folic acid group than in the pregnant + homocysteine group ($P < 0.05$) (Figure 4).

DISCUSSION

In our study, methionine was not administered because methionine induced moderate homocysteinemia and may affect overall protein synthesis. We created hyperhomocysteinemia by intraperitoneal injection of homocysteine directly. We determined that hyperhomocysteinemia in pregnant rats elicited several symptoms of preeclampsia, namely hypertension, proteinuria, fetal intrauterine growth restriction and an increase in nonviable pups. However, the symptoms of hypertension were also seen in hyperhomocysteinemia nonpregnant rats. Interestingly, this phenomenon was much more evident in pregnant rats than in nonpregnant rats. The level of homocysteine in nonpregnant rats was a little lower than that in pregnant rats treated with homocysteine although the dose of DL-homocysteine was not different between the two groups. This phenomenon is consistent with the

study of
Powers *et al* [19], which suggested that severe hyperhomocysteinemia may increase susceptibility to preeclampsia by causing endothelial dysfunction during pregnancy.

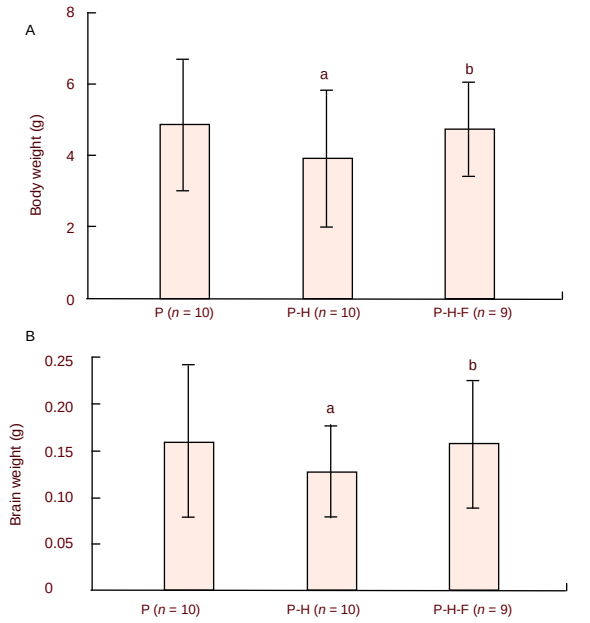

Figure 4 Effect of folic acid on body weight (A) and brain weight (B) of rat pups.

Data are expressed as mean ± SD. [a]*P* < 0.05, *vs.* P rats. [b]*P* < 0.05, *vs.* P-H rats. The differences among groups were compared by analysis of variance and *post-hoc* least significant difference test.

C: Nonpregnant control; C-H: nonpregnant + homocysteine group; P: pregnant control; P-H: pregnant + homocysteine; P-H-F: pregnant + homocysteine + folic acid.

Folic acid is a coenzyme in the production of nucleic acids and therefore is required by all cells for growth[6]. An adequate cellular folate supply may play an important role in the implantation and development of the placenta. Folate may also reduce the risk of developing preeclampsia by improving endothelial function at both placental and systemic levels, directly or indirectly by its effect on lowering plasma homocysteine level[20-21]. In our study, preeclampsia-like symptoms were improved, such as systolic blood pressure and proteinuria in the hyperhomocysteinemia rats treated with folic acid. Additionally, folic acid treatment did not result in a decrease in average pup weight. The supplementation of folic acid improved the brain weight of pups, which might help us to explore the hypothesis that folic acid would probably contribute to brain protection.

There are several limitations to this study. The most notable is that all animal models of preeclampsia are flawed. In our models, the systolic blood pressure change, although significant, was not as high as that seen in other animal model studies[22]. The symptoms of hypertension were also seen in hyperhomocysteinemia nonpregnant rats. This is likely due to a fact that the dosage and persistence time of DL-homocysteine in rats was different from other studies.

Chandler *et al* [23] found hyperhomocysteinemia had no significant effect on systolic blood pressure in pregnant rats, although they increased plasma concentration of homocysteine to levels comparable to humans with preeclampsia. These findings are not consistent with our study where a 10 day homocysteine treatment in pregnant rats resulted in an 8–9 fold increase in plasma homocysteine but evident effect on blood pressure.

The other question this study raises is that folic acid improves pup brain weight. However, the underlying mechanism for this protection remains unknown. To answer this question, we determined the neuroprotective effects of folic acid on cortical neurons induced by hyperhomocysteinemia in pregnant rats, which may be related to their anti-apoptotic properties[24].

Taken together, our findings show that hyperhomocysteinemia during pregnancy produces preeclampsia-like symptoms in rats and this model may provide a means for better understanding the underlying mechanisms that lead to preeclampsia in some women. Dietary supplementation of folic acid might have greater significance in the protection against preeclampsia. Hence, our study strongly supports the hypothesis that folate supplementation is also beneficial to fetal development.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This study was performed at the Laboratory Animal Center, General Hospital of Chinese PLA from August 2009 to June 2010.

Materials

For mating purposes, ten male Wistar rats and fifty female Wistar rats, weighing 200–250 g, were purchased from the General Hospital of Chinese PLA (Shenyang, China) and included in all experiments. Rats were maintained on a 12-hour light/dark cycle and had free access to standard chow and water *ad libitum*. All studies conformed with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals[25].

Methods

Establishment of hyperhomocysteinemic rat model and folic acid administration

To establish the hyperhomocysteinemic rat model, DL-homocysteine (Sigma, St. Louis, MO, USA) was daily administered by intraperitoneal injection at a dose of 200 mg/kg from day 10 to day 19 of gestation in the pregnant + homocysteine, nonpregnant + homocysteine and pregnant + homocysteine + folic acid groups. Folic acid was intragastrically administered at a dose of 20 mg/kg from day 10 to day 19 of gestation 2 hours after DL-homocysteine injection in the pregnant + homocysteine + folic acid group. On corresponding days, nonpregnant control and pregnant rats received saline administration. Rats were sacrificed on day 20 of gestation and nonpregnant rats were sacrificed on the corresponding day.

Determination of systolic blood pressure

Systolic arterial blood pressure was measured by tail-cuff plethysmography[26]. Animals were warmed to 32°C and systolic arterial blood pressure measurements were performed by experienced investigators using a model 59 amplifier (IITC Inc. Woodland Hills, CA, USA).

Determination of proteinuria

One day before euthanasia, 24-hour urine was collected from the animals housed individually in metabolic cages in the absence of food to eliminate contamination of urinary protein measurements by fallen food particles. Urinary creatinine concentration was measured using the micro pyrogallol red method (Total Protein Kit, Sigma, St. Louis, MO, USA). Urinary creatinine concentration was determined using a Nova electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA, USA). The results were expressed as a ratio of urinary protein concentration to urinary creatinine concentration.

Plasma homocysteine

At the end of the measurements of the above-mentioned parameters, 1 mL of blood was collected through a catheter in the right carotid. The plasma was centrifuged and homocysteine was separated by high performance liquid chromatography and measured by colorimetry[27]. Briefly, blood samples of 1 mL were collected into Vacutainer tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA) and immediately centrifuged at 1 000 × *g* for 10 minutes at 4°C. Plasma samples (100 μL) or solutions mixed with 10 μL internal standard (2-mercaptoethylamine, 2.0 mM), were treated with 10 μL 10% (v/v) tri-n-butylphosphine in imethylformamide at 4°C for 30 minutes. Subsequently, 100 μL supernatant was transferred into a solution containing 20 μL 1.55 M sodium hydroxide, 250 μL 0.125 M borate buffer (pH 9.5), and 100 μL 1.0 mg/mL 4-Fluoro-7-sulfamoylbenzofurazan solution. The resulting mixture was incubated at 60°C for 30 minutes to accomplish derivatization of plasma thiols. High performance liquid chromatography was performed with a Hewlett-Packard Model 1090 Series II system (Analytical Instrument Recycle Inc., Golden, CO, USA) with an autosampler. Separation was carried out at ambient temperature on an analytical column, Supelco LC-18-DB (150 mm × 4.6 mm I.D., 5 μm) with a Supelcosil LC-18 guard column (20 mm × 4.6 mm I.D., 5 μm). Fluorescence intensities were measured spectrophotometrically (Hewlett-Packard Model 1046A) at an excitation wavelength of 385 nm and emission wavelength of 515 nm. The peak area of the chromatographs was quantified with a Hewlett-Packard 3392 integrator (Analytical Instrument Recycle Inc.). The analytical column was eluted with 0.1 M potassium dihydrogenphosphate buffer (pH 2.1) containing 6% acetonitrile (v/v) as a mobile phase with a flow rate of 2.0 mL/min.

Neonatal parameters

The pups were delivered *via* hysterotomy after the blood was collected on day 20 of gestation. Pups (in one of the litters from each group) were weighed by electronic scale (Hengx-HXW, Taiwan) and sacrificed by cervical dislocation. Pup brains were collected and the wet weight was weighed.

Statistical analysis

All data were statistically analyzed using SPSS 11.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. The differences among groups were compared by analysis of variance and the *post-hoc* least significant difference test. Statistical significance was set at $P < 0.05$.

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Author contributions: Jun Wang participated in the study design, experimental execution, data analysis, interpretation and manuscript writing. Jing Ge participated in the study design, data analysis, interpretation, and assisted in proof reading of the manuscript. Meijing Ma assisted in experimental execution and manuscript revision. All authors read and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: The protocol was approved by the Institu-

tional Animal Care and Use Committee of China Medical University, China.

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