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

Jun Wang¹, Yan Cui², Jing Ge¹, Meijing Ma³

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.
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\(^9\,\,^10\). 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 \( (P = 0.015) \).
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).

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

Plasma homocysteine levels in the pregnant and non-pregnant 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).

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
Folic acid is a coenzyme in the production of nucleic acids and therefore is required by all cells for growth. 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. Dietary supplementation of folic acid might have greater significance in the protection against preeclampsia. Hence, our study strongly supports the hypothesis that folic acid 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.
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 (IIITC 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 μM), were treated with 10 μL 10% (v/v) tri-n-butylphosphine in imethylfor-
ternal Animal Care and Use Committee of China Medical University, China.

REFERENCES

[1] Valdiviezo C, Garovic VD, Ouyang P. Preeclampsia and hypertensive disease in pregnancy: their contributions to cardiovascular risk. Clin Cardiol. 2012;35(3):160-165.

[2] Tsai IH, Chen CP, Sun FJ, et al. Associations of the pre-pregnancy body mass index and gestational weight gain with pregnancy outcomes in Taiwanese women. Asia Pac J Clin Nutr. 2012;21(1):82-7.

[3] Roberts JM, Speer P. Antioxidant therapy to prevent preeclampsia. Semin Nephrol. 2004;24(6):557-64.

[4] Molvarec A, Ito M, Shima T, et al. Decreased proportion of peripheral blood vascular endothelial growth factor-expressing T and natural killer cells in preeclampsia. Am J Obstet Gynecol. 2010;203(6):567.e1-8.

[5] Munjal C, Tyagi N, Lominadze D, et al. Matrix metalloproteinase-9 in homocysteine-induced intestinal microvascular endothelial paracellular and transcellular permeability. J Cell Biochem. 2012;113(4):1159-1169.

[6] Kang RX, Zhang JJ. A natural squamosamide derivative FLZ inhibits homocysteine-induced rat brain microvascular endothelial cells dysfunction. Biochem Biophys Res Commun. 2012;417(4):1176-1181.

[7] McCully KS. Chemical pathology of homocysteine. V. Thioretinamide, thioretinaco, and cystathionine synthase function in degenerative diseases. Ann Clin Lab Sci. 2011;41(4):301-314.

[8] Sen U, Mishra PK, Tyagi N, et al. Homocysteine to hydrogen sulfide or hypertension. Cell Biochem Biophys. 2010;57(2-3):49-58.

[9] Khosrowbeygi A, Ahmadvand H. Circulating levels of homocysteine in preeclamptic women. Bangladesh Med Res Councl Bull. 2011;37(3):106-109.

[10] Mislanova C, Martsenyuk Y, Huppertz B, et al. Placental markers of folate-related metabolism in preeclampsia. Reproduction. 2011;142(3):467-476.

[11] Hague WM. Homocysteine and pregnancy. Best Pract Res Clin Obstet Gynaecol. 2003;17(3):459-69.

[12] Davison RL, Hoffmann DS, Butz GM, et al. Discovery of a spontaneous genetic mouse model of preeclampsia. Hypertension. 2002;39(2 Pt 2):337-342.

[13] Cross JC. The genetics of pre-eclampsia: a feto-placental or maternal problem? Clin Genet. 2003;64(2):96-103.

[14] Granger JP, LaMarca BB, Cockrell K, et al. Reduced uterine perfusion pressure (RUPP) model for studying cardiovascular-renal dysfunction in response to placental ischemia. Methods Mol Med. 2006;122:383-392.

[15] Mutter WP, Karumanchi SA. Molecular mechanisms of preeclampsia. Microvasc Res. 2008;75(1):1-8.

[16] Bodnar LM, Tang G, Ness RB, et al. Periconceptional multivitamin use reduces the risk of preeclampsia. Am J Epidemiol. 2006;164(5):470-477.

[17] Fekete K, Berti C, Cetin I, et al. Perinatal folate supply: relevance in health outcome parameters. Matern Child Nutr. 2010;6 Suppl 2:23-38.

[18] Olthof MR, Bots ML, Katan MB, et al. Effect of folic acid and betaine supplementation on flow-mediated dilation: a randomized, controlled study in healthy volunteers. PLoS Clin Trials. 2006 Jun;1(2):e10.

[19] Powers RW, Gandy RE, Lykins DL, et al. Moderate hyperhomocysteinemia decreases endothelial-dependent vasorelaxation in pregnant but not nonpregnant mice. Hypertension. 2004;44(3):327-333.

[20] Stanger O, Wonisch W. Enzymatic and non-enzymatic antioxidative effects of folic acid and its reduced derivatives. Subcell Biochem. 2012;56:131-161.

[21] Qipshidze N, Metreveli N, Lominadze D, et al. Folic acid improves acetylcholine-induced vasoconstriction of coronary vessels isolated from hyperhomocysteinemic mice: an implication to coronary vasospasm. J Cell Physiol. 2011;226(10):2712-20.

[22] Alexander BT, Kassab SE, Miller MT, et al. Reduced uterine perfusion pressure during pregnancy in the rat is associated with increases in arterial pressure and changes in renal nitric oxide. Hypertension. 2001;37(4):1191-1195.

[23] Chandler DL, Linas MT, Reckelhoff JF, et al. Effects of hyperhomocysteinemia on arterial pressure and nitric oxide production in pregnant rats. Am J Hypertens. 2009;22(10):1115-1119.

[24] Yu HL, Li L, Zhang XH, et al. Neuroprotective effects of genistein and folic acid on apoptosis of rat cultured cortical neurons induced by beta-amyloid 31-35. Br J Nutr. 2009;102(5):655-662.

[25] The Ministry of Science and Technology of the People’s Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

[26] Whitesell SE, Hoff JB, Vollmer AP, et al. Comparison of simultaneous measurement of mouse systolic arterial blood pressure by radiotelemetry and tail-cuff methods. Am J Physiol Heart Circ Physiol. 2004;286(6):H2408-2415.

[27] Li N, Chen YF, Zou AP. Implications of hyperhomocysteinemia in glomerular sclerosis in hypertension. Hypertension. 2002;39(2 Pt 2):443-448.

(Edited by Shang LX, Yu DW/Song LP)