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Folic acid supplementation during pregnancy protects against lipopolysaccharide-induced neural tube defects in mice

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HIGHLIGHTS

- Folic acid supplementation during pregnancy prevented LPS-induced NTDs.
- Folic acid attenuated LPS-induced GSH depletion in maternal liver and placenta.
- Folic acid alleviated LPS-induced inflammatory cytokines.
- Folic acid inhibited LPS-induced JNK phosphorylation in placenta.
- Folic acid inhibited LPS-induced placental NF-κB activation.

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ABSTRACT

Folic acid is a water-soluble B-complex vitamin. Increasing evidence demonstrates that physiological supply of folic acid during pregnancy prevents folic acid deficiency-related neural tube defects (NTDs). Previous studies showed that maternal lipopolysaccharide (LPS) exposure caused NTDs in rodents. The aim of this study was to investigate the effects of high-dose folic acid supplementation during pregnancy on LPS-induced NTDs. Pregnant mice were intraperitoneally injected with LPS (20 μg/kg/d) from gestational day (GD) 8 to GD12. As expected, a five-day LPS injection resulted in 19.96% of fetuses with NTDs. Interestingly, supplementation with folic acid (3 mg/kg/d) during pregnancy significantly alleviated LPS-induced NTDs. Additionally, folic acid significantly attenuated LPS-induced fetal growth restriction and skeletal malformations. Additional experiment showed that folic acid attenuated LPS-induced glutathione (GSH) depletion in maternal liver and placentas. Moreover, folic acid significantly attenuated LPS-induced expression of placental MyD88. Additionally, folic acid inhibited LPS-induced c-Jun NH2-terminal kinase (JNK) phosphorylation and nuclear factor kappa B (NF-κB) activation in placentas. Correspondingly, folic acid significantly attenuated LPS-induced tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in placentas, maternal serum and amniotic fluid. In conclusion, supplementation with high-dose folic acid during pregnancy protects against LPS-induced NTDs through its anti-inflammatory and anti-oxidative effects.

1. Introduction

Lipopolysaccharide (LPS) is a toxic component of cell wall in Gram-negative bacteria and is widely present in the digestive tracts of humans and animals (Jacob et al., 1977). Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal inflammatory diseases and excess alcohol intake are known to increase permeability of LPS from gastrointestinal tract into blood (Zhou et al., 2003). According to an earlier report, LPS has also been detected in the cervical mucus and vaginal fluid from pregnant women with bacterial vaginosis (Platz-Christensen et al., 1993). LPS has been associated with adverse developmental outcomes, such as embryonic resorption, fetal death, intrauterine growth restriction (IUGR), skeletal development retardation and preterm delivery in rodents (Haddad et al., 1995; Silver et al., 1995; Buhimschi et al., 2003; Xu et al., 2005). According to an earlier report, maternal

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low-dose LPS exposure at gestational day (GD) 8 resulted in neural tube defects (NTDs) in golden hamsters (Lanning et al., 1983). Moreover, subcutaneous LPS injection on GD8 led to fetal malformation in mice (Carey et al., 2003; Chua et al., 2006). A recent report from our lab demonstrated that injection with a low dose of LPS (20 μg/kg/d, equivalent to 1/1000 LD_{50}) to pregnant mice daily from GD8 to GD12 caused NTDs including exencephaly and encephalomeningocele (Zhao et al., 2008).

The mechanism of LPS-induced developmental toxicity has been extensively studied. Several studies have demonstrated that excess reactive oxygen species (ROS) partially contributes to LPS-induced fetal death, IUGR and NTDs in mice (Zhao et al., 2008). Some antioxidants, such as melatonin, N-acetylcysteine and ascorbic acid, could protect against LPS-induced IUGR and skeletal development retardation (Xu et al., 2005; Chen et al., 2006a,b). On the other hand, inflammatory cytokines are also involved in LPS-induced developmental toxicity including preterm delivery, fetal death and IUGR. Indeed, maternal LPS exposure during pregnancy significantly elevated the level of inflammatory cytokines in maternal serum, amniotic fluid and placentas (Xu et al., 2006a, 2007; Ning et al., 2008). Moreover, pentoxifylline, a specific TNF-α synthetic inhibitor, and TNFR2-IgG, the antagonist of TNF-α signaling, protected against LPS-induced fetal death, IUGR and fetal neurogenesis impairment (Carpentier et al., 2011).

Folic acid is a water-soluble B-complex vitamin, which plays a crucial role on one-carbon metabolism for physiological DNA synthesis and cell division (Fox and Stover, 2008). Indeed, folic acid deficiency during pregnancy results in the occurrence of NTDs (Smithells et al., 1976). US Preventive Services Task Force recommends that all women planning or capable of pregnancy take a daily physiological supplement containing 0.4–0.8 mg of folic acid for the prevention of folic acid deficiency-related NTDs (US Preventive Services Task Force, 2009). Several experiments have demonstrated that supplementation with high-dose folic acid (3 mg/kg/d) is effective in preventing diabetes- or heat-induced NTDs in mice, which suggests that folic acid can prevent some specific teratogens-induced NTDs by other mechanisms (Shin and Shiota, 1999; Oyama et al., 2009). Nevertheless, whether supplementation with folic acid during pregnancy protects against LPS-induced NTDs is unknown.

In the present study, we investigated the effects of supplementation with high-dose folic acid during pregnancy on LPS-induced NTDs. Our results showed that folic acid supplementation during pregnancy protected against LPS-induced NTDs in mice. We demonstrated that protection of folic acid against LPS-induced NTDs is attributed to its anti-inflammatory and anti-oxidative effects.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and folic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nuclear factor-kappa B p65 (NF-κB p65, SC-372), phosphor-inhibitor of kappa B (p-IκB, Ser 32, SC-8404), phosphor-c-Jun NH2-terminal kinase (p-JNK, Thr 183/Tyr 185, SC-6254), c-Jun NH2-terminal kinase (JNK, SC-572) and Lamin A/C (SC-6215) antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). MyD88 antibody (#4283S) was from Cell Signaling Technology (Danvers, MA, USA). β-Actin antibody was from Boster Bio-Technology Co. Ltd. (Wuhan, China). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL, USA). TRI reagent (TR 118) for the isolation of total RNA was from Molecular Research Center, Inc. (Cincinnati, OH, USA). RNase-free DNase was from Promega Corporation (Madison, WI, USA). All the other reagents were from Sigma or as indicated in the specified methods.

2.2. Animals and treatments

The ICR mice (8–10 week-old; male mice: 28–30 g; female mice: 24–26 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. (Wilmington, MA, USA). The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. For mating purposes, four females were housed overnight with two males starting at 9:00 P.M. Females were checked by 7:00 A.M. the next morning, and the presence of a vaginal plug was designated as GD0.

Table 1
Primers for real-time RT-PCR.

Genes	Sequences	Sizes (bp)
gapdh	Forward: 5′-ACCCCAGCAAGGACACTGAGCAAG-3′ Reverse: 5′-GGCCCCTCCTGTTATTATGGGGGT-3′	109
tnf-α	Forward: 5′-CCCTCCTGGCCAACGGCATG-3′ Reverse: 5′-TCGGGGCAGCCTTGTCCCTT-3′	109
il-1β	Forward: 5′-GCCTCGTGCTGTCGGACCCATAT-3′ Reverse: 5′-TCCTTTGAGGCCCAAGGCCACA-3′	143
il-6	Forward: 5′-AGACAAAGCCAGAGTCCTTCAGAGA-3′ Reverse: 5′-GCCACTCCTTCTGTGACTCCAGC-3′	146

Pregnant mice were divided into four groups ($n = 16$ per group) randomly. In the LPS alone group, the pregnant mice were intraperitoneally (i.p.) injected with LPS (20 μg/kg) daily from GD8 to GD12. In the LPS + folic acid (FA) group, the pregnant mice were administered folic acid (3 mg/kg) by gavage 1 h before LPS injection. The doses of folic acid used in the present study referred to others (Shin and Shiota, 1999; Cipollone et al., 2009; Oyama et al., 2009). In the control group, the pregnant mice were i.p. injected with normal saline (NS) daily from GD8 to GD12. In the FA alone group, the pregnant mice were administered folic acid (3 mg/kg) by gavage 1 h before NS injection. All dams were anesthetized with phenobarbital sodium (50 mg/kg) and sacrificed by cervical dislocation on GD18. Then gravid uterine weight was recorded. For each litter, the number of live fetuses, dead fetuses and resorption sites was counted. Live fetuses and placentas in each litter were weighed. Live fetuses were examined for NTDs including exencephaly and encephalomeningocele. Crown-rump length was measured. All fetuses were subsequently evaluated the supraoccipital ossification and skeletal malformations.

To investigate the effects of folic acid supplementation on LPS-induced oxidative stress and inflammation, the pregnant mice were divided into four groups ($n = 12$ per group) randomly. The pregnant mice were treated as above methods. All dams were anesthetized with phenobarbital sodium and sacrificed at 2 h after the last LPS injection on GD12. Maternal serum and amniotic fluid were collected for measurement of inflammatory cytokines (TNF-α, IL-1β and IL-6). Some placentas were collected for real-time RT-PCR and immunoblot. Other placentas and maternal liver were collected for measurement of glutathione (GSH) content.

All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

2.3. Skeletal examination and evaluation

The fetuses were eviscerated and macerated in 1% KOH solution containing alizarin red. After a week of staining and corrosion, stained fetuses were again cleared of residual muscle and subsequently placed in 0.1% KOH solution containing 2% glycerol for 3 days and then placed in 50% glycerin solution for subsequent skeletal examination. The supraoccipital ossification and sternum and rib malformations were determined. Each fetus was evaluated the supraoccipital ossification according to supraoccipital ossification score method. The method is described as follows: 1, well ossified; 2, bilateral occipital ossification points linked together, and the width of joint less than one-third of the bilateral width; 3, biggish bilateral occipital ossification points observed but not linked together; 4, small bilateral or unilateral occipital ossification points observed but not linked together; and 5, no brain case or brain case intact but no supraoccipital ossification. Sternum malformations included sternum lack, sternum split, fishtail sternum. Rib malformations included fused, supernumerary, missing, bifurcated, or wavy/bulbous ribs.

2.4. Isolation of total RNA and real-time RT-PCR

Total RNA was extracted using TRI reagent according to the manufacturer's instructions. The purity of RNA was assessed according to the ratio of absorbance at 260 nm and 280 nm. RNase-free DNase (Promega, Madison, WI, USA) was used to remove genomic DNA. The cDNA was synthesized from 2 μg of total RNA using Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). Real-time RT-PCR was performed with a LightCycler® 480 SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) using gene-specific primers. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on each individual sample as an internal positive-control standard. As shown in Table 1, the primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China), according to sequence designs previously described by others (Chen et al., 2012). The comparative C_T-method was used to

determine the amount of target. Relative expression levels of mRNAs were calculated by the comparative threshold cycle method using GAPDH as an internal control. The purity of PCR products was verified by melting curves. All RT-PCR experiments were performed in triplicate.

2.5. Immunoblot

Total lysate from placenta was prepared by homogenizing 50 mg placenta tissue in 300 μl lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulphate, 1 mM phenylmethylsulfonyl fluoride) supplemented with a cocktail of protease inhibitors (Roche). For nuclear protein extraction, total lysate from placenta was suspended in hypotonic buffer and then kept on ice for 15 min. The suspension was then mixed with detergent and centrifuged for 30 s at 14,000 × *g*. The nuclear pellet obtained was resuspended in complete lysis buffer in the presence of the protease inhibitor cocktail, incubated for 30 min on ice, and centrifuged for 10 min at 14,000 × *g*. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. For immunoblot, same amount of protein (40–80 μg) was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were incubated for 2 h with the following antibodies: MyD88, p-JNK, JNK, p-IκB and NF-κB p65. For total proteins, β-actin was used as a loading control. For nuclear protein, Lamin A/C was used as a loading control. After being washed in DPBS containing 0.05% Tween-20 four times, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse antibody for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20, followed by signal development using an ECL detection kit.

2.6. Enzyme-linked immunosorbent assay

Commercial enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) kits were used to determine the levels of TNF-α, IL-1β and IL-6 in maternal serum and amniotic fluid according to manufacturer's protocol.

2.7. Determination of glutathione content

Samples of maternal liver and placentas were homogenized in iced-cold normal saline. The homogenates were centrifuged at 4000 × *g* for 10 min at 4 °C. The supernatants were collected and protein concentrations were determined with the BCA protein assay reagents. Proteins of 0.4 ml of homogenates were precipitated by the addition of 0.2 ml of 20% trichloroacetic acid. The protein precipitate was separated from the remaining solution by centrifugation at 4000 × *g* at 4 °C for 10 min. The supernatant (0.1 ml) was combined with 4.4 ml of 300 mM Na_2HPO_4 buffer. Then 0.5 ml of dithio-bis-nitrobenzoic acid (DTNB) (0.04%, w/v; 40 mg DTNB in 100 ml of 1% sodium citrate) was added to the blank and sample. The absorbance of the sample was determined at 412 nm within 5 min. GSH content was determined using a calibration curve prepared with reference standard. All GSH assay experiments were performed in triplicate. GSH content was indicated as nanomole GSH per milligram of protein.

2.8. Statistical analysis

The litter was considered the unit for statistical comparison among different groups. Fetal mortality was calculated in litter and then averaged in each group. For fetal weight, crown-rump length, placenta weight and skeletal evaluation, the means were calculated in litter and then averaged in each group. Quantified data were presented as means ± S.E.M. Values of $P < 0.05$ were considered statistically significant. ANOVA and the Fisher's least significant difference (LSD) test were performed to determine significant differences between the treated animals and the control.

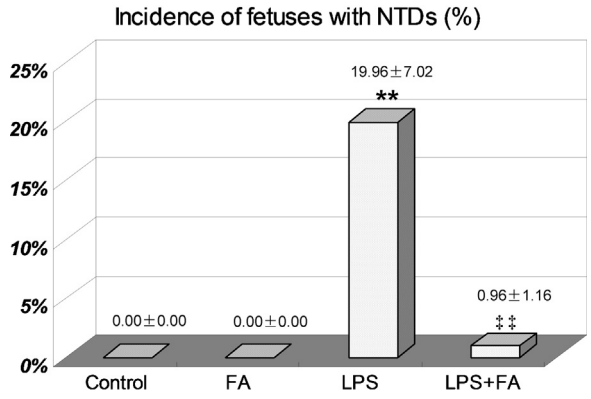

Fig. 1. Effects of folic acid supplementation on LPS-induced NTDs. In LPS alone group, the pregnant mice were i.p. injected with LPS (20 μg/kg) daily from GD8 to GD12. In LPS + FA group, the pregnant mice were administered with folic acid (3 mg/kg) by gavage 1 h before LPS injection. In control group, the pregnant mice were i.p. injected with NS daily from GD8 to GD12. In FA alone group, the pregnant mice were administered with folic acid (3 mg/kg) by gavage 1 h before NS injection. All pregnant mice were sacrificed on GD18. Live fetuses were examined for NTDs. Fetal malformations were calculated in litter and then averaged in each group. Data were presented as means ± S.E.M. $^{**}P < 0.01$ vs. the control. $^{‡‡}P < 0.01$ vs. LPS group.

3. Results

3.1. Effects of folic acid on LPS-induced NTDs

Folic acid had no effect on fodder consumption and weight gain of the pregnant mice (data not shown). No abortion was observed. No dams died throughout the pregnancy. All pregnant mice completed pregnancy. The number of litters, resorptions per litter, live fetuses per litter and dead fetuses per litter was presented in Table 2. There was no significant difference in the number of resorptions per litter and live fetuses per litter among different groups. As shown in Table 2, the number of dead fetuses per litter was slightly increased in LPS-treated group. Interestingly, folic acid had little effect on LPS-induced fetal death (Table 2). The effects of folic acid on LPS-induced NTDs were then analyzed. As shown in Table 2, a five-day LPS injection resulted in 50% (8/16) of litters with NTDs. Among dams injected with LPS, 19.96% of fetuses per litter were with NTDs (Fig. 1). Interestingly, the number of litters with NTDs was significantly reduced to 6.25% (1/16) when pregnant mice were pretreated with folic acid (Table 2). Correspondingly, the incidence of fetuses with NTDs dropped to 0.96% in dams pretreated with folic acid before LPS (Fig. 1).

Table 2
Fetal outcomes among different groups.

	Control	FA	LPS	LPS + FA
Number of litters (*n*)	16	16	16	16
Number of litters with NTDs (*n*)	0	0	8	1
Resorptions per litter (*n*)	0.5 ± 0.2	0.4 ± 0.2	0.6 ± 0.2	0.7 ± 0.2
Dead fetuses per litter (*n*)	0.1 ± 0.1	0.3 ± 0.1	1.0 ± 0.2*	0.9 ± 0.5
Numbers of live fetuses (*n*)	219	218	205	206
Live fetuses per litter (*n*)	13.7 ± 0.5	13.6 ± 0.8	12.8 ± 0.8	12.9 ± 0.6
Fetal weight (g)	1.42 ± 0.01	1.41 ± 0.02	1.30 ± 0.03**	1.40 ± 0.02‡‡
Crown-rump length (cm)	2.46 ± 0.02	2.44 ± 0.01	2.29 ± 0.03**	2.36 ± 0.03‡
Placenta weight (g)	0.098 ± 0.003	0.095 ± 0.003	0.085 ± 0.003**	0.091 ± 0.002

Note. In LPS group, the pregnant mice were injected with LPS (20 μg/kg, i.p.) daily from GD8 to GD12. In LPS + FA group, the pregnant mice were administered folic acid (3 mg/kg) by gavage 1 h before LPS injection. All quantitative data were expressed as means ± S.E.M.

* $P < 0.05$ as compared with the control.
** $P < 0.01$ as compared with the control.
‡ $P < 0.05$ as compared with LPS group.
‡‡ $P < 0.01$ as compared with LPS group.

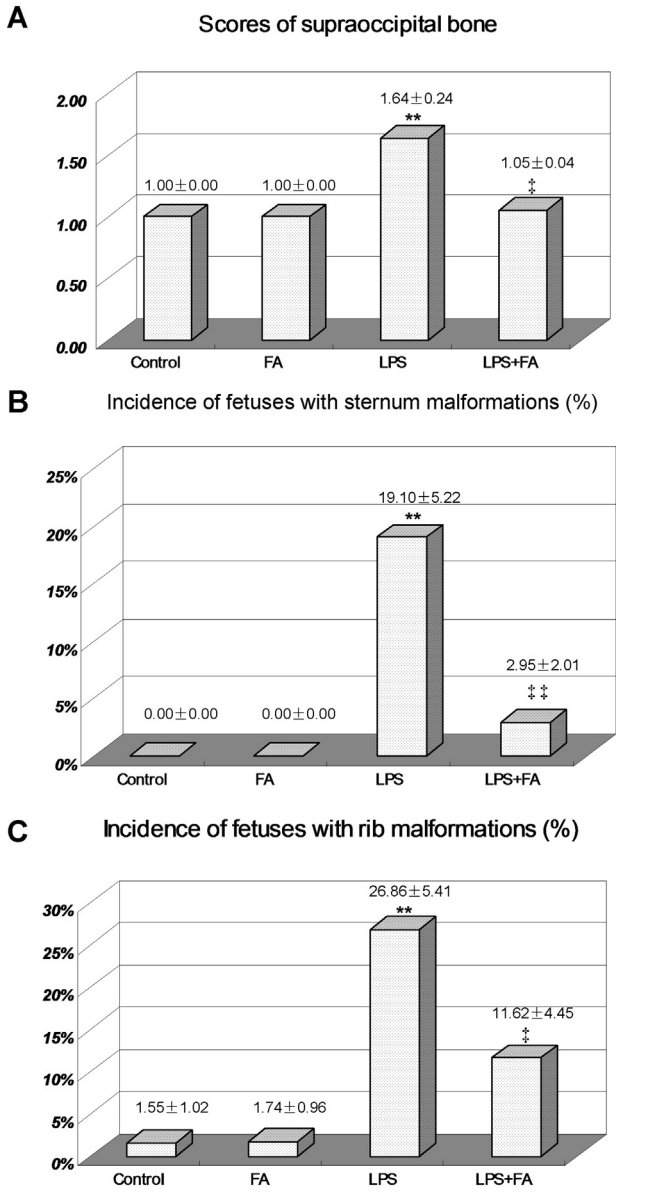

Fig. 2. Effects of folic acid supplementation on LPS-induced skeletal malformations. In LPS alone group, the pregnant mice were i.p. injected with LPS (20 μg/kg) daily from GD8 to GD12. In LPS + FA group, the pregnant mice were administered with folic acid (3 mg/kg) by gavage 1 h before LPS injection. In control group, the pregnant mice were i.p. injected with NS daily from GD8 to GD12. In FA alone group, the pregnant mice were administered with folic acid (3 mg/kg) by gavage 1 h before NS injection. All pregnant mice were sacrificed on GD 18. Live fetuses were examined for skeletal malformations. (A) Supraoccipital bone scores: 1, well ossified; 5, completely unossified. (B) The incidence of fetus with sternum malformations. (C) The incidence of fetus with rib malformations. Fetal malformations were calculated in litter and then averaged in each group. Data were presented as means ± S.E.M. $^{**}P < 0.01$ vs. the control. $^{\ddagger\ddagger}P < 0.01$ vs. LPS group.

3.2. Effects of folic acid on LPS-induced skeletal malformations

As shown in Fig. 2A, maternal LPS exposure retarded fetal supraoccipital ossification. Supplementation with folic acid during pregnancy significantly attenuated LPS-induced skeletal development retardation. The effects of folic acid supplementation on LPS-induced sternum and rib malformations are presented in Fig. 2B and C. Maternal LPS exposure also resulted in 19.10% of fetuses with sternum malformation and 26.86% of fetuses with rib malformation. The incidence of fetus with sternum and rib malformations

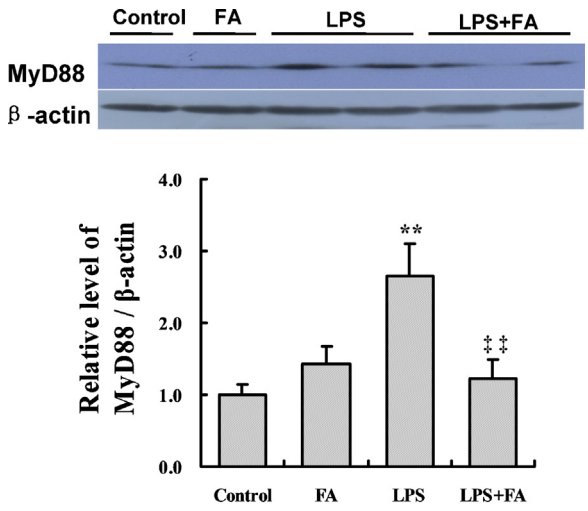

Fig. 3. Effects of folic acid supplementation on LPS-induced expression of placental MyD88. All pregnant mice except controls were i.p. injected with LPS (20 μg/kg) daily from GD8 to GD12. In LPS + FA group, the pregnant mice were administered with folic acid (3 mg/kg) by gavage 1 h before LPS injection. Placentas were collected at 2 h after the last LPS injection. Placental MyD88 was measured using immunoblot. A representative gel for MyD88 (upper panel) and β-actin (lower panel) was shown. All experiments were repeated for three times. All data were presented as means ± S.E.M. $^{**}P < 0.01$ vs. the control. $^{\ddagger\ddagger}P < 0.01$ vs. LPS group.

declined to 2.95% and 11.62%, respectively, in dams pretreated with folic acid before LPS.

3.3. Effects of folic acid on LPS-induced IUGR

As shown in Table 2, a five-day LPS injection markedly reduced fetal weight and crown-rump length. Interestingly, fetal weight rose from 1.30 ± 0.03 g in LPS-treated dams to 1.40 ± 0.02 g in dams pretreated with folic acid before LPS. Correspondingly, folic acid supplementation during pregnancy alleviated LPS-induced reduction of fetal crown-rump length ($P < 0.05$).

3.4. Effects of folic acid on LPS-induced placental MyD88 expression

As shown in Fig. 3, the level of placental MyD88 was significantly increased by 2.7-fold with LPS treatment. Folic acid alone had no effect on the expression of placental MyD88. Of interest, expression of MyD88 diminished 2.2-fold in placentas of mice with folic acid pretreatment before LPS relative to placentas in LPS-treated mice.

3.5. Effects of folic acid on LPS-induced placental JNK phosphorylation

As shown in Fig. 4, the level of phosphorylated JNK was significantly increased 4.9-fold in placentas of mice injected with LPS relative to saline-treated mice. Treatment with folic acid alone had no significant effect on JNK phosphorylation in placentas. Interestingly, pretreatment with folic acid before LPS induced a 2-fold decrease in the level of phosphorylyated JNK compared to the level of LPS-induced placental JNK phosphorylation.

3.6. Effects of folic acid on LPS-induced placental NF-κB activation

As shown in Fig. 5A, the level of phosphorylated IκB was significantly increased in placentas of mice injected with LPS. Correspondingly, LPS treatment significantly elevated the level of placental nuclear NF-κB p65 (Fig. 5B). Interestingly, the level of placental phosphorylated IκB was significantly decreased in dams

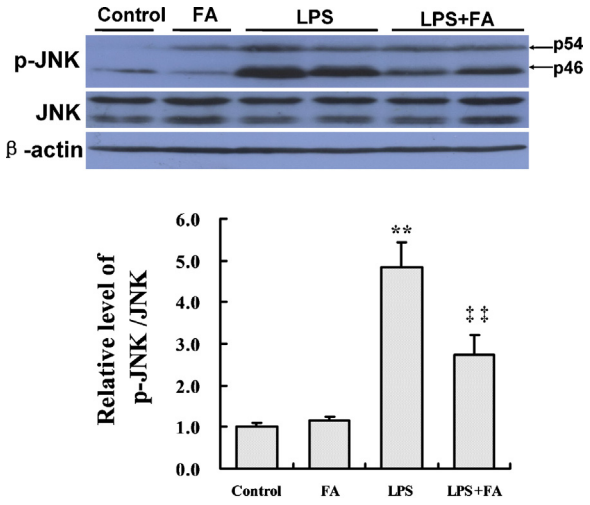

Fig. 4. Effects of folic acid supplementation on placental JNK phosphorylation. All pregnant mice except controls were i.p. injected with LPS (20 μg/kg) daily from GD8 to GD12. In LPS + FA group, the pregnant mice were administered with folic acid (3 mg/kg) by gavage 1 h before LPS injection. Placentas were collected at 2 h after the last LPS injection. Placental p-JNK was measured using immunoblot. A representative gel for p-JNK (upper panel) and JNK (lower panel) was shown. All experiments were repeated for three times. All data were presented as means ± S.E.M. $^{**}P < 0.01$ vs. the control. $^{‡}P < 0.05$ vs. LPS group.

pretreated with folic acid before LPS (Fig. 5A). In addition, supplementation with folic acid significantly attenuated LPS-evoked nuclear translocation of NF-κB p65 in placentas (Fig. 5B).

3.7. Effects of folic acid on LPS-induced inflammatory cytokines

As shown in Fig. 6A–C, the expression of TNF-α, IL-1β and IL-6 mRNA was significantly increased in placentas of mice injected with LPS. Folic acid pretreatment significantly inhibited LPS-induced upregulation of TNF-α, IL-1β and IL-6 mRNA in placentas. The effects of folic acid on LPS-induced inflammatory cytokines in maternal serum were presented in Fig. 6D–F. As expected, LPS significantly increased the levels of TNF-α, IL-1β and IL-6 in maternal serum. Interestingly, the levels of TNF-α, IL-1β and IL-6 were significantly decreased in maternal serum of mice pretreated with folic acid before LPS. As shown in Fig. 6G–I, the levels of TNF-α, IL-1β and IL-6 were significantly increased in amniotic fluid of mice injected with LPS. Folic acid pretreatment significantly alleviated LPS-induced inflammatory cytokines in amniotic fluid.

3.8. Effects of folic acid on LPS-induced GSH depletion

As shown in Fig. 7A, LPS significantly reduced GSH content in maternal liver (Fig. 7A). In addition, maternal LPS exposure slightly reduced GSH content in placentas (Fig. 7B). Interestingly, when pregnant mice were pretreated with folic acid, the levels of GSH in maternal liver and placentas rebounded (Fig. 7A and B).

4. Discussion

The present study showed that daily intraperitoneal injection with low dose LPS during organogenesis (GD8-GD12) caused NTDs and skeletal malformations in mice. These results are consistent with previous studies (Zhao et al., 2008). Physiological supply of folic acid (0.4–0.8 mg/d) during pregnancy is recommended to reduce the risk of folic acid deficiency-related NTDs (Czeizel and Dudas, 1992; US Preventive Services Task Force, 2009). In this study, we investigated the effect of supplementation with a high dose of folic acid on LPS-induced NTDs in mice. Interestingly, we found that supplementation with folic acid during pregnancy significantly reduced the incidence of LPS-induced NTDs from 19.96% to 0.96%. In addition, folic acid supplementation significantly attenuated LPS-induced skeletal malformations. These results suggest that folic acid supplementation during pregnancy

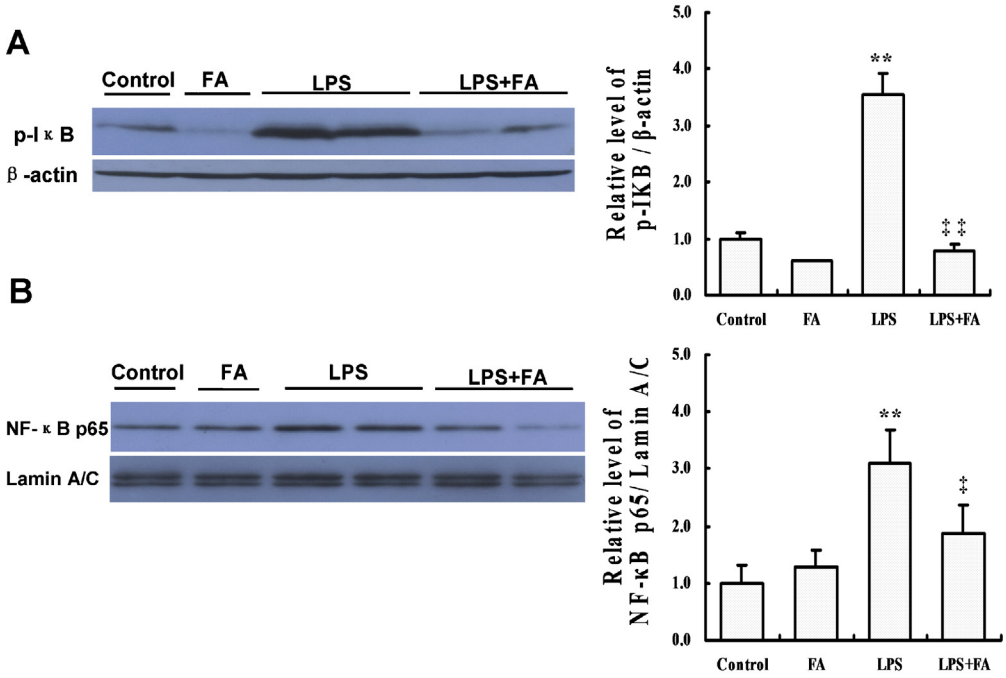

Fig. 5. Effects of folic acid supplementation on LPS-induced placental NF-κ B activation. All pregnant mice except controls were i.p. injected with LPS (20 μg/kg) daily from GD8 to GD12. In LPS + FA group, the pregnant mice were administered with folic acid (3 mg/kg) by gavage 1 h before LPS injection. Placentas were collected at 2 h after the last LPS injection. (A) Placental p-IκB was measured using immunoblot. A representative gel for p-IκB (upper panel) and β-actin (lower panel) was shown. (B) The nuclear fractions were prepared from placentas. NF-κB p65 was measured using Western blot. A representative gel for p65 (upper panel) and Lamin A/C (lower panel) was shown. All experiments were repeated for three times. All data were presented as means ± S.E.M. $^{**}P < 0.01$ vs. the control. $^{‡}P < 0.05$, $^{‡‡}P < 0.01$ vs. LPS group.

prevents not only folic acid deficiency-related NTDs but also LPS-induced NTDs, which expands the protective effects of folic acid against NTDs.

An earlier report showed that maternal LPS exposure enhanced the level of 4-hydroxy-2-nonenal (HNE)-modified protein in placentas (Ejima et al., 2000). A recent study found that maternal LPS exposure upregulated the expression of placental heme oxygenase (HO)-1, a marker of oxidative stress (Zhang et al., 2007). The present study showed that the levels of GSH content in maternal liver and placentas were decreased when the pregnant mice were injected with LPS. Indeed, it has been demonstrated that excess ROS production is involved in LPS-induced teratogenicity in mice (Xu et al., 2006b; Zhao et al., 2008). Several studies showed that folic acid has an antioxidant effect (Hwang et al., 2011; Sarna et al., 2012).

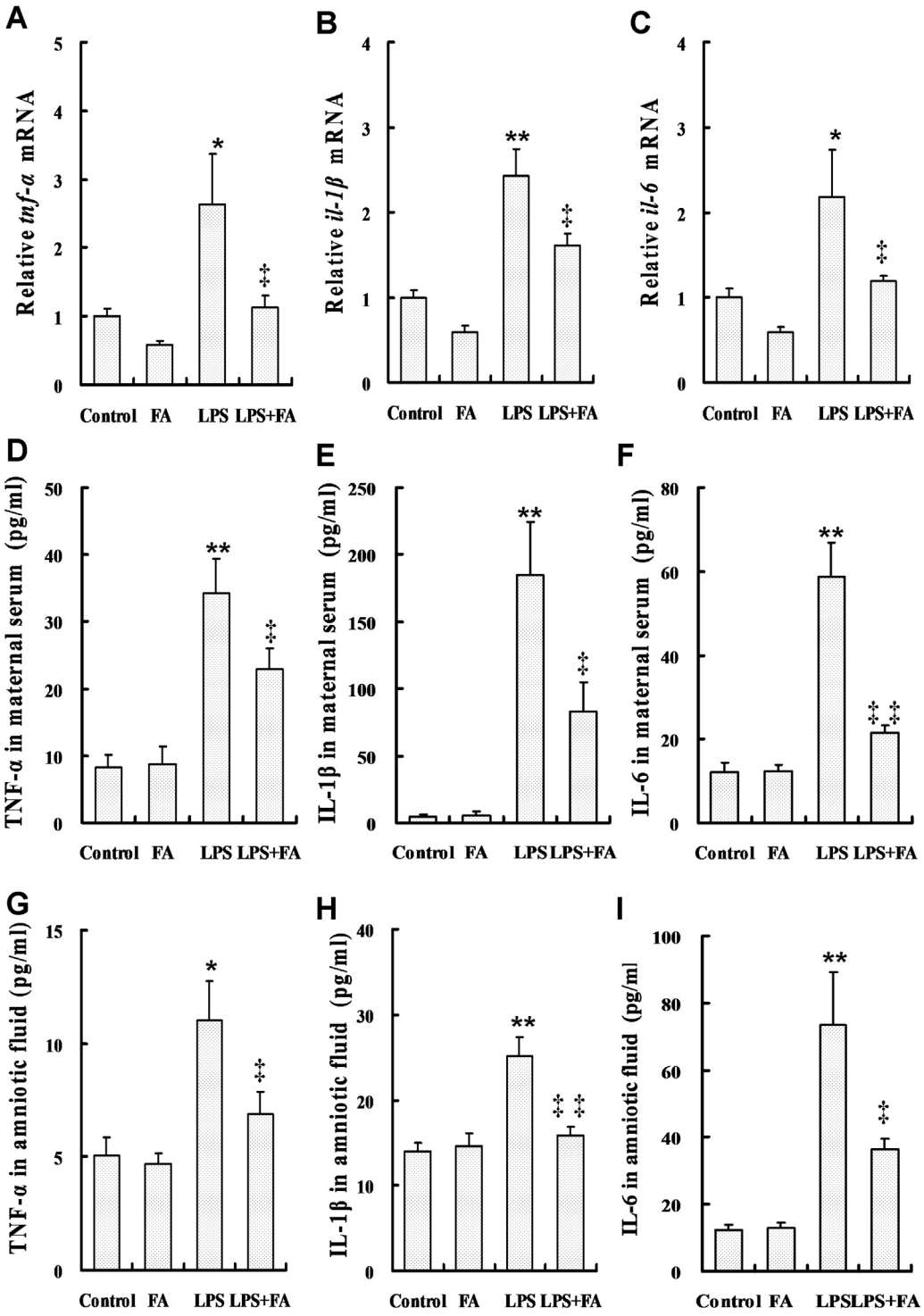

Fig. 6. Folic acid supplementation during pregnancy inhibits LPS-evoked inflammatory cytokines. (A–C) Placentas were collected at 2 h after the last LPS injection. TNF-α, IL-1β and IL-6 mRNA were measured using real-time RT-PCR. (A) TNF-α. (B) IL-1β. (C) IL-6. (D–F) Maternal sera were collected at 2 h after the last LPS injection. TNF-α, IL-1β and IL-6 in maternal serum were measured using ELISA. (D) TNF-α. (E) IL-1β. (F) IL-6. (G–I) Amniotic fluid was collected at 2 h after the last LPS injection. TNF-α, IL-1β and IL-6 in amniotic fluid were measured using ELISA. (G) TNF-α. (H) IL-1β. (I) IL-6. All data were presented as means ± S.E.M. (n = 6 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control. $^{‡}P < 0.05$, $^{‡‡}P < 0.01$ vs. LPS group.

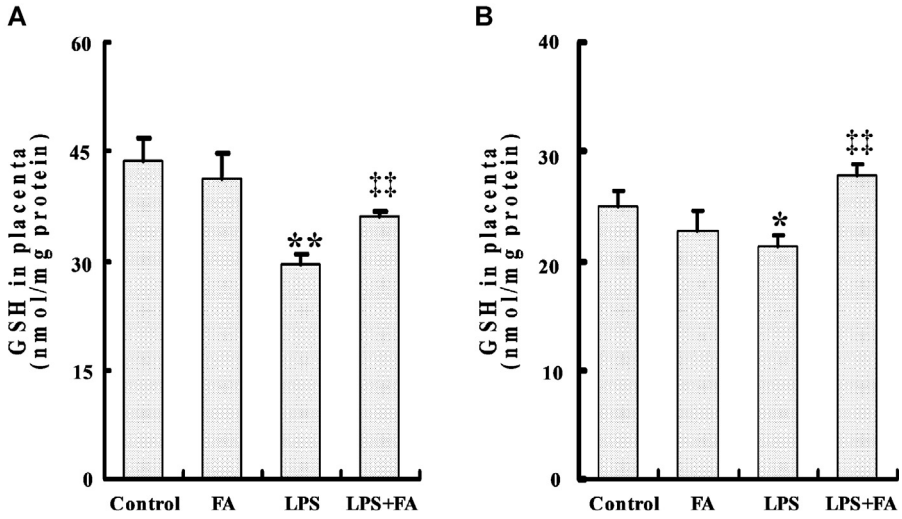

Fig. 7. Folic acid supplementation during pregnancy attenuates LPS-induced GSH depletion in maternal liver and placentas. Maternal liver and placentas were excised at 2 h after the last LPS injection. (A) GSH in maternal liver. (B) GSH in placentas. All experiments were repeated for three times. Data were presented as means ± S.E.M. ($n = 12$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control. $^{‡‡}P < 0.01$ vs. LPS group.

The present study found that supplementation with folic acid during pregnancy alleviated LPS-induced GSH depletion in maternal liver and placentas. These results suggest that the protective effect of folic acid against LPS-induced NTDs is partially attributed to its antioxidant effect.

Increasing evidence demonstrates that inflammatory cytokines are involved in LPS-induced preterm delivery, fetal death and IUGR (Leazer et al., 2002; Xu et al., 2006a; Carpentier et al., 2011). Indeed, maternal LPS exposure during pregnancy stimulated inflammatory cytokine production (Gayle et al., 2004; Xu et al., 2007). Folic acid has an antiinflammatory effect. According to an earlier report, folic acid reduced the release of chemokines from peripheral blood mononuclear cells in hyperhomocysteinemia subjects (Holven et al., 2002). A recent study showed that folic acid inhibited LPS-stimulated inflammatory cytokines in macrophages (Feng et al., 2011). The present study showed that supplementation with folic acid during pregnancy significantly attenuated LPS-induced upregulation of TNF-α, IL-1β and IL-6 mRNA in placentas. Moreover, supplementation with folic acid during pregnancy significantly alleviated LPS-induced release of TNF-α, IL-1β and IL-6 in maternal serum and amniotic fluid. These results suggest that the protection of folic acid against LPS-induced developmental toxicity might, at least partially, be attributed to its anti-inflammatory effects.

The MAPK signaling is one of the most important signaling cascades that regulate LPS-induced inflammatory genes (Guha and Mackman, 2001). The present study showed that the level of placental phosphorylated JNK was significantly increased when pregnant mice were injected with LPS from GD8 to GD12. A recent report demonstrates that folic acid inhibits LPS-induced inflammatory response by suppressing JNK phosphorylation in macrophages (Feng et al., 2011). The present study found for the first time that folic acid supplementation during pregnancy significantly attenuated LPS-induced placental JNK phosphorylation. A recent report from our laboratory showed that maternal LPS exposure during late gestational stage (GD15) activated placental MAPK p38 and ERK signaling (Chen et al., 2012). The present study showed that maternal LPS exposure during middle gestational stage had little effect on placental MAPK p38 and ERK signaling (data not shown). Taken together, these results suggest that folic acid suppresses LPS-induced inflammatory cytokines partially due to its inhibition of placental JNK phosphorylation.

NF-κB plays a central role in LPS-induced inflammatory cytokines (Baeuerle and Henkel, 1994). Under unstimulated conditions, NF-κB is usually retained in the cytoplasm by binding to IκB. IκB phosphorylation causes translocation of NF-κB to the nucleus (Baldwin Jr., 1996). The present study showed that the level of phosphorylated IκB was significantly increased in the placentas of mice injected with LPS. Correspondingly, the level of nuclear NF-κB p65 was significantly increased in the placentas of mice injected with LPS. An *in vitro* study showed that folic acid abrogated LPS-induced NF-κB activation and subsequent TNF-α release in macrophages (Feng et al., 2011). The present study showed that folic acid significantly attenuated LPS-evoked placental IκB phosphorylation. Moreover, folic acid supplementation during pregnancy significantly inhibited LPS-induced placental nuclear NF-κB p65 translocation. These results suggest that folic acid supplementation during pregnancy suppresses LPS-induced inflammation through its inhibition of placental NF-κB activation.

Increasing evidence indicates that TLR4 modulates placental NF-κB signaling (Arce et al., 2009). MyD88 is the key signaling adaptor that is involved in TLR4-mediated NF-κB activation (Layoun et al., 2012). The present study showed that the level of MyD88 was significantly increased in the placentas when the pregnant mice were injected with LPS from GD8 to GD12. Interestingly, folic acid significantly attenuated LPS-induced upregulation of placental MyD88. These results suggest that folic acid could inhibit MyD88-dependent TLR4 signaling in the placentas. Thus, additional work is required to determine how folic acid modulates placental TLR4 signaling and subsequent inflammatory response.

In this study, we have shown that high-dose folic acid can protect mouse embryos against LPS-induced NTDs in mice. Generally, it is deemed that physiological supply of folic acid during pregnancy functions by ameliorating folic acid deficiency-related NTDs (Czeizel and Dudas, 1992; US Preventive Services Task Force, 2009). Interestingly, our results demonstrate that high-dose folic acid supplementation during pregnancy inhibits LPS-induced inflammation and oxidative stress, which is involved in the underlying mechanism of LPS-induced NTDs. These results suggest that the protection of high-dose folic acid against NTDs extends beyond folic acid deficiency through its anti-inflammatory and anti-oxidative effects. Given these results, supplementation with a high dose of folic acid has clinical application value in prevention of LPS-induced NTDs, which is in line with several studies suggesting that pregnant

women with higher risk of NTDs can benefit more from high doses of folic acid (4–5 mg/d) (MRC Vitamin Study Research Group, 1991; Wald et al., 2001).

In summary, the present results allow us to reach the following conclusions. First, folic acid supplementation during pregnancy can efficiently prevent LPS-induced NTDs. Second, the protection of folic acid against LPS-induced NTDs might be, at least partially, attributed to its anti-inflammatory and anti-oxidative effects. We conclude that supplementation with folic acid during pregnancy has potential preventive and therapeutic utilities for preventing not only folic acid-deficiency-related NTDs but also LPS-induced teratogenicity.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

Arce, R.M., Barros, S.P., Wacker, B., Peters, B., Moss, K., Offenbacher, S., 2009. Increased TLR4 expression in murine placentas after oral infection with periodontal pathogens. Placenta 30, 156–162.

Baeuerle, P.A., Henkel, T., 1994. Function and activation of NF-kappa B in the immune system. Annual Review of Immunology 12, 141–179.

Baldwin Jr., A.S., 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annual Review of Immunology 14, 649–683.

Buhimschi, I.A., Buhimschi, C.S., Weiner, C.P., 2003. Protective effect of N-acetylcysteine against fetal death and preterm labor induced by maternal inflammation. American Journal of Obstetrics and Gynecology 188, 203–208.

Carey, L.C., Berbee, P.L., Coyle, P., Philcox, J.C., Rofe, A.M., 2003. Zinc treatment prevents lipopolysaccharide-induced teratogenicity in mice. Birth Defects Research Part A: Clinical and Molecular Teratology 67, 240–245.

Carpentier, P.A., Dingman, A.L., Palmer, T.D., 2011. Placental TNF-alpha signaling in illness-induced complications of pregnancy. American Journal of Pathology 178, 2802–2810.

Chen, Y.H., Xu, D.X., Wang, J.P., Wang, H., Wei, L.Z., Sun, M.F., Wei, W., 2006a. Melatonin protects against lipopolysaccharide-induced intrauterine fetal death and growth retardation in mice. Journal of Pineal Research 40, 40–47.

Chen, Y.H., Xu, D.X., Zhao, L., Wang, H., Wang, J.P., Wei, W., 2006b. Ascorbic acid protects against lipopolysaccharide-induced intrauterine fetal death and intrauterine growth retardation in mice. Toxicology 217, 39–45.

Chen, Y.H., Zhao, M., Chen, X., Zhang, Y., Wang, H., Huang, Y.Y., Wang, Z., Zhang, Z.H., Zhang, C., Xu, D.X., 2012. Zinc supplementation during pregnancy protects against lipopolysaccharide-induced fetal growth restriction and demise through its anti-inflammatory effect. Journal of Immunology 189, 454–463.

Chua, J.S., Rofe, A.M., Coyle, P., 2006. Dietary zinc supplementation ameliorates LPS-induced teratogenicity in mice. Pediatric Research 59, 355–358.

Cipollone, D., Carsetti, R., Tagliani, A., Rosado, M.M., Borgiani, P., Novelli, G., D'Amati, G., Fumagalli, L., Marino, B., Businaro, R., 2009. Folic acid and methionine in the prevention of teratogen-induced congenital defects in mice. Cardiovascular Pathology 18, 100–109.

Czeizel, A.E., Dudas, I., 1992. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. New England Journal of Medicine 327, 1832–1835.

Ejima, K., Koji, T., Tsuruta, D., Nanri, H., Kashimura, M., Ikeda, M., 2000. Induction of apoptosis in placentas of pregnant mice exposed to lipopolysaccharides: possible involvement of Fas/Fas ligand system. Biology of Reproduction 62, 178–185.

Feng, D., Zhou, Y., Xia, M., Ma, J., 2011. Folic acid inhibits lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages by suppressing MAPKs and NF-kappaB activation. Inflammation Research 60, 817–822.

Fox, J.T., Stover, P.J., 2008. Folate-mediated one-carbon metabolism. Vitamins and Hormones 79, 1–44.

Gayle, D.A., Beloosesky, R., Desai, M., Amidi, F., Nunez, S.E., Ross, M.G., 2004. Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology 286, R1024–R1029.

Guha, M., Mackman, N., 2001. LPS induction of gene expression in human monocytes. Cellular Signalling 13, 85–94.

Haddad, E.K., Duclos, A.J., Baines, M.G., 1995. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. Journal of experimental Medicine 182, 1143–1151.

Holven, K.B., Aukrust, P., Holm, T., Ose, L., Nenseter, M.S., 2002. Folic acid treatment reduces chemokine release from peripheral blood mononuclear cells in hyperhomocysteinemic subjects. Arteriosclerosis, Thrombosis, and Vascular biology 22, 699–703.

Hwang, S.Y., Siow, Y.L., Au-Yeung, K.K., House, J.O.K., 2011. Folic acid supplementation inhibits NADPH oxidase-mediated superoxide anion production in the kidney. American Journal of Physiology: Renal Physiology 300, F189–F198.

Jacob, A.I., Goldberg, P.K., Bloom, N., Degenshein, G.A., Kozinn, P.J., 1977. Endotoxin and bacteria in portal blood. Gastroenterology 72, 1268–1270.

Lanning, J.C., Hilbelink, D.R., Chen, L.T., 1983. Teratogenic effects of endotoxin on the golden hamster. Teratogenesis, Carcinogenesis, and Mutagenesis 3, 145–149.

Layoun, A., Huang, H., Calve, A., Santos, M.M., 2012. Toll-like receptor signal adaptor protein MyD88 is required for sustained endotoxin-induced acute hypoferremic response in mice. American Journal of Pathology 180, 2340–2350.

Leazer, T.M., Barbee, B., Ebron-McCoy, M., Henry-Sam, G.A., Rogers, J.M., 2002. Role of the maternal acute phase response and tumor necrosis factor alpha in the developmental toxicity of lipopolysaccharide in the CD-1 mouse. Reproductive Toxicology 16, 173–179.

MRC Vitamin Study Research Group, 1991. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. Lancet 338, 131–137.

Ning, H., Wang, H., Zhao, L., Zhang, C., Li, X.Y., Chen, Y.H., Xu, D.X., 2008. Maternally-administered lipopolysaccharide (LPS) increases tumor necrosis factor alpha in fetal liver and fetal brain: its suppression by low-dose LPS pretreatment. Toxicology Letters 176, 13–19.

Oyama, K., Sugimura, Y., Murase, T., Uchida, A., Hayasaka, S., Oiso, Y., Murata, Y., 2009. Folic acid prevents congenital malformations in the offspring of diabetic mice. Endocrine Journal 56, 29–37.

Platz-Christensen, J.J., Mattsby-Baltzer, I., Thomsen, P., Wiqvist, N., 1993. Endotoxin and interleukin-1 alpha in the cervical mucus and vaginal fluid of pregnant women with bacterial vaginosis. American Journal of Obstetrics and Gynecology 169, 1161–1166.

Sarna, L.K., Wu, N., Wang, P., Hwang, S.Y., Siow, Y.L.O.K., 2012. Folic acid supplementation attenuates high fat diet induced hepatic oxidative stress via regulation of NADPH oxidase. Canadian Journal of Physiology and Pharmacology 90, 155–165.

Shin, J.H., Shiota, K., 1999. Folic acid supplementation of pregnant mice suppresses heat-induced neural tube defects in the offspring. Journal of Nutrition 129, 2070–2073.

Silver, R.M., Edwin, S.S., Trautman, M.S., Simmons, D.L., Branch, D.W., Dudley, D.J., Mitchell, M.D., 1995. Bacterial lipopolysaccharide-mediated fetal death. Production of a newly recognized form of inducible cyclooxygenase (COX-2) in murine decidua in response to lipopolysaccharide. Journal of Clinical Investigation 95, 725–731.

Smithells, R.W., Sheppard, S., Schorah, C.J., 1976. Vitamin deficiencies and neural tube defects. Archives of Disease in Childhood 51, 944–950.

US Preventive Services Task Force, 2009. Folic acid for the prevention of neural tube defects: U.S. Preventive Services Task Force recommendation statement. Annals of Internal Medicine 150, 626–631.

Wald, N.J., Law, M.R., Morris, J.K., Wald, D.S., 2001. Quantifying the effect of folic acid. Lancet 358, 2069–2073.

Xu, D.X., Chen, Y.H., Wang, H., Zhao, L., Wang, J.P., Wei, W., 2005. Effect of N-acetylcysteine on lipopolysaccharide-induced intrauterine fetal death and intrauterine growth retardation in mice. Toxicological Sciences 88, 525–533.

Xu, D.X., Chen, Y.H., Wang, H., Zhao, L., Wang, J.P., Wei, W., 2006a. Tumor necrosis factor alpha partially contributes to lipopolysaccharide-induced intrauterine fetal growth restriction and skeletal development retardation in mice. Toxicology Letters 163, 20–29.

Xu, D.X., Chen, Y.H., Zhao, L., Wang, H., Wei, W., 2006b. Reactive oxygen species are involved in lipopolysaccharide-induced intrauterine growth restriction and skeletal development retardation in mice. American Journal of Obstetrics and Gynecology 195, 1707–1714.

Xu, D.X., Wang, H., Ning, H., Zhao, L., Chen, Y.H., 2007. Maternally administered melatonin differentially regulates lipopolysaccharide-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain. Journal of Pineal Research 43, 74–79.

Zhang, C., Li, X.Y., Zhao, L., Wang, H., Xu, D.X., 2007. Lipopolysaccharide (LPS) upregulates the expression of heme oxygenase-1 in mouse placenta. Placenta 28, 951–957.

Zhao, L., Chen, Y.H., Wang, H., Ji, Y.L., Ning, H., Wang, S.F., Zhang, C., Lu, J.W., Duan, Z.H., Xu, D.X., 2008. Reactive oxygen species contribute to lipopolysaccharide-induced teratogenesis in mice. Toxicological Sciences 103, 149–157.

Zhou, Z., Wang, L., Song, Z., Lambert, J.C., McClain, C.J., Kang, Y.J., 2003. A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-alpha production. American Journal of Pathology 163, 1137–1146.