Available online at www.sciencedirect.com
ScienceDirect
Toxicology 234 (2007) 167–175
TOXICOLOGY
www.elsevier.com/locate/toxicol

# Effects of low-dose lipopolysaccharide (LPS) pretreatment on LPS-induced intra-uterine fetal death and preterm labor

De-Xiang Xu [a,b,*], Hua Wang [a], Lei Zhao [a], Huan Ning [a], Yuan-Hua Chen [a], Cheng Zhang [a]

[a] *Department of Toxicology, Anhui Medical University, Hefei 230032, PR China*
[b] *Department of Microbiology and Parasitology, Anhui Medical University, Hefei 230032, PR China*

Received 22 January 2007; received in revised form 20 February 2007; accepted 21 February 2007
Available online 24 February 2007

**Abstract**

Lipopolysaccharide (LPS) has been associated with adverse developmental outcome, including embryonic resorption, intra-uterine fetal death (IUFD), intra-uterine growth retardation (IUGR) and preterm delivery in rodents. The purpose of the present study was to investigate whether administration of a low-dose LPS to the pregnant mice induce a reduced sensitivity to subsequent high-dose LPS-induced IUFD and preterm labor. We found that LPS-induced IUFD was obviously attenuated when the pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 24 h before high-dose LPS (120 μg/kg, i.p.). Consistent with its protective effect, when administered 24 h before high-dose LPS, low-dose LPS pretreatment obviously inhibited the releases of tumor necrosis factor alpha (TNF-α) in maternal serum and amniotic fluid and attenuated LPS-induced placental lipid peroxidation and GSH depletion. However, when administered 4 h before high-dose LPS, low-dose LPS pretreatment did not induced a reduced sensitivity to subsequent high-dose LPS-induced release of TNF-α in maternal serum and amniotic fluid. Actually, low-dose LPS pretreatment 4 h before high-dose LPS worsened LPS-induced oxidative stress in mouse placenta and increased nitric oxide production in maternal serum and amniotic fluid. Correspondingly, low-dose LPS pretreatment 4 h before high-dose LPS aggravated LPS-induced IUFD. Taken together, these results indicate that whether a low-dose LPS exposure during pregnancy produce LPS hyporesponsiveness depends on the interval between the two doses of LPS. When administered 24 h before high-dose LPS, a low-dose LPS pretreatment induces a reduced sensitivity to subsequent high-dose LPS-induced IUFD, TNF-α production and oxidative stress.


*Keywords:* Lipopolysaccharide; Tumor necrosis factor alpha; Developmental toxicity; Intra-uterine fetal death; Lipopolysaccharide hyporesponsiveness

## 1. Introduction

Lipopolysaccharide (LPS) is a toxic component of cell walls of Gram-negative bacteria and is widely present in the digestive tracts of humans and animals (Jacob et al., 1997). Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal distress and alcohol drinking often increase permeability of LPS from gastrointestinal tract into blood (Fukui et al., 1991). In human, Gram-negative bacterial infections are a recognized cause of fetal loss and preterm labor (Romero et al., 1988). LPS has been associated with adverse developmental outcome, including embryonic resorption, intra-uterine fetal death (IUFD), intra-uterine growth retardation (IUGR) and preterm

* Corresponding author at: Department of Toxicology, Anhui Medical University, Hefei 230032, PR China. Tel.: +86 551 5167923; fax: +86 551 5161179.
*E-mail address:* xudex@mail.hf.ah.cn (D.-X. Xu).

0300-483X/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2007.02.010

delivery in rodents (Silver et al., 1995; Ogando et al., 2003; Buhimschi et al., 2003).

In the LPS model, tumor necrosis factor alpha (TNF-α) is one of the major mediators leading to embryonic resorption, IUFD, IUGR and preterm delivery. Numerous studies showed that LPS resulted in increase in TNF-α in maternal serum, amniotic fluid and placenta (Bell et al., 2004; Vizi et al., 2001; Gayle et al., 2004; Chen et al., 2005). TNF-α has been associated with LPS-induced embryonic resorption, abortion and preterm labor (Silver et al., 1994; Gendron et al., 1990). Several studies have demonstrated that TNF-α contributes to LPS-induced IUFD and IUGR (Leazer et al., 2002; Xu et al., 2006a,b). Nitric oxide is another important mediator contributing to LPS-induced adverse developmental outcome. An earlier study showed that maternal LPS exposure induced the expression of inducible nitric oxide synthase (iNOS) in decidual and myometrial cells and increased nitric oxide production in decidual and uterine (Ogando et al., 2003). In addition, aminoguanidine (AG), an inhibitor of iNOS activity, reversed LPS-induced embryonic resorption and abortion (Athanassakis et al., 1999). Prostaglandins are also involved in LPS-induced embryotoxicity. An earlier study showed that maternally administered LPS induced $COX_2$ expression and resulted in an elevation of decidual eicosanoid production, followed by a dose-dependent increase in embryo death (Silver et al., 1995). $COX_2$ suppressors protected mice against LPS-induced IUFD and preterm delivery (Gross et al., 2000; Sakai et al., 2001). Recently, we found that reactive oxygen species (ROS) were, at least in part, involved in LPS-induced IUFD and IUGR (Xu et al., 2006a,b). Several antioxidants protected mice against LPS-induced IUFD and IUGR. (Xu et al., 2005; Chen et al., 2006).

Pretreatment with LPS is shown to induce a reduced sensitivity to subsequent challenge of LPS. This phenomenon is termed LPS tolerance or LPS hyporesponsiveness. LPS tolerance was observed in vivo febrile response and escape from lethality as well as in vitro with a reduced production of inflammatory cytokines in response to a secondary stimulation with LPS (Erroi et al., 1993; Medvedev et al., 2000). An in vitro study showed that LPS tolerance in mouse peritoneal macrophages is associated with downregulation of toll-like receptor 4 (TLR4) expression (Nomura et al., 2000). Soon afterwards, another study found that intratracheal LPS induced a rapid reduction in whole lung TLR4 mRNA, an effect which is also observed in recovered alveolar macrophages (Fan et al., 2002). However, it is unclear whether a low-dose LPS exposure during pregnancy also produce a LPS hyporesponsiveness.

The purpose of the present study was to investigate whether administration of a low-dose LPS to the pregnant mice induce a reduced sensitivity to subsequent high-dose LPS-induced IUFD and preterm labor. In addition, the present study also assessed the effect of low-dose LPS pretreatment on LPS-induced TNF-α, oxidative stress and nitric oxide production.

## 2. Materials and methods

### 2.1. Chemicals

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) was purchased from Sigma Chemical Co. (St. Louis, MO). 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. The animals were allowed free access to food and water at all times and were maintained on a 12-h light:12-h 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 gestational day (gd) 0. The present study consisted of two separate experiments.

Experiment 1. The pregnant mice were divided into four groups randomly. In Group A, the pregnant mice received an intraperitoneal (i.p.) injection of LPS (120 μg/kg) on gd 15. In Group B1, the pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 h before high-dose LPS (120 μg/kg, i.p.). In Group B2, the pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 24 h before high-dose LPS (120 μg/kg, i.p.). The control mice received saline (Group C). The animals were observed closely for evidence of preterm labor and delivery. The remaining dams were sacrificed on gd 18 and gravid uterine weights were recorded. For each litter, the number of live fetuses, dead fetuses and resorption sites were counted. Live fetuses in each litter were weighed and crown-rump lengths were measured.

Experiment 2. The pregnant mice were divided into four groups randomly. In Group A, the pregnant mice received an intraperitoneal injection of LPS (120 μg/kg) on gd 15. In Group B1, the pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 h before high-dose LPS (120 μg/kg, i.p.). In Group B2, the pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 24 h before high-dose LPS (120 μg/kg, i.p.). The control mice received saline (Group C). Twelve pregnant mice from each group were sacrificed 1.5 h after high-dose LPS treatment. Maternal serum and amniotic fluid were harvested for measurement of TNF-α and IL-10. Twelve preg-

nant mice from each group were sacrificed 6 h after high-dose LPS. Placentas were collected for measurement of glutathione (GSH) and thiobarbituric acid-reactive substance (TBARS). Maternal serum and amniotic fluid were harvested for measurement of nitrite plus nitrate.

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.

#### 2.2.1. Lipid peroxidation assay

Lipid peroxidation was quantified by measuring TBARS as described previously (Ohkawa et al., 1979). Tissue was homogenized in 9 volumes of 50 mmol/L Tris–HCl buffer (pH 7.4) containing 180 mmol/L KCl, 10 mmol/L EDTA and 0.02% butylated hydroxytoluene. To 0.2 mL of the tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.9% thiobarbituric acid and 0.6 mL of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95 °C for 1 h. After cooling on ice, 1.0 mL of distilled water and 5.0 mL of butanol/pyridine mixture (15:1, v/v) were added and vortexed. After centrifugation at 10,000 *g* for 10 min, absorbance of the resulting lower phase was determined at 532 nm. The TBARS concentration was calculated using 1,1,5,5-tetraethoxypropane as standard.

#### 2.2.2. Determination of GSH content

GSH was determined by the method of Griffith (1980). Proteins of 0.4 mL liver homogenates were precipitated by the addition of 0.4 mL of a metaphosphoric acid solution. After 40 min, the protein precipitate was separated from the remaining solution by centrifugation at 5000 rpm at 4 °C for 5 min. Four hundred microliters of the supernatant was combined with 0.4 mL of 300 mM $Na_2HPO_4$, and the absorbance at 412 nm was read against a blank consisting of 0.4 mL supernatant plus 0.4 mL $H_2O$. Then, 100 μL DTNB (0.02%, w/v; 20 mg DTNB in 100 mL of 1% sodium citrate) was added to the blank and sample. Absorbance of the sample was read against the blank at 412 nm. The GSH content was determined using a calibration curve prepared with an authentic sample. GSH values were expressed as nmol $mg^{-1}$ protein. Protein content was measured according to the method of Lowry et al. (1951).

### 2.3. Measurement of TNF-α and IL-10

Commercial ELISA (R&D Systems) kits were used to determine levels of TNF-α in maternal serum and amniotic fluid according to the manufacturer's protocol. Briefly, samples were pipetted in wells precoated with specific antibody for mouse TNF-α and allowed to incubate for 2 h. After wells were rinsed to remove all unbound substance, an enzyme-linked antibody specific for mouse TNF-α was added to wells for 2 h. After wells were rinsed to remove all unbound enzyme-linked antibody, a substrate solution was added to wells for 30 min to yield a colored product that was quantified by optical density readings at 450 nm. The reaction was stopped and the optical density was measured at 450 nm using a Universal microplate reader (Bio-Tek Instruments, Inc.).

### 2.4. Analysis of nitrite plus nitrate concentration

The stable end products of L-arginine-dependent nitric oxide synthesis, nitrate plus nitrite, were measured in maternal serum and amniotic fluid using a colorimetric method based on the Griess reaction (Grisham et al., 1996). Briefly, 20 μL of sample were mixed with 20 μL of 0.31 M phosphate buffer, pH 7.5, 10 μL of 0.1 mM FAD, 10 μL of 1 mM NADPH, 10 mL of nitrate reductase (10 units/mL) and 30 μL of water in a 96-well plate. The reaction was allowed to proceed for 1 h in the dark. The percent conversion of nitrate to nitrite was 98%. To each sample, 1 μL of lactate dehydrogenase (1500 units/mL) and 10 μL of 100 mM pyruvic acid were added and incubated for 15 min at 37°. The samples were then mixed with an equivalent volume of Griess reagent and incubated for an additional 10 min at room temperature. Nitrite levels were determined colorimetrically at 550 nm with a Universal microplate reader (Bio-Tek Instruments, Inc.) and a sodium nitrite standard curve.

### 2.5. Statistical analysis

The litter was considered the unit for statistical comparison among different groups. For fetal weight and crown-rump length, the means were calculated per litter and then averaged per group. Quantified data were expressed as means ± S.E.M. at each point. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences among different groups. For preterm delivery or fetal death, chi-square analyses was used with the Fisher exact correction when necessary. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effects of low-dose LPS pretreatment on LPS-induced preterm labor and IUFD

In the control group, there were no pregnant mice delivered before gd 18. Preliminary results showed that administration of low-dose LPS (10 μg/kg, i.p.) on gd 14 or gd 15 did not induce preterm labor (data not shown). The effects of LPS on preterm labor are presented in Fig. 1A. Results showed that administration of a single dose LPS (120 μg/kg, i.p.) on gd 15 resulted in 26.7% (4/15) pregnant mice delivered before gd 18. In the low-dose LPS pretreatment group, only 6.7% (1/15) pregnant mice delivered before gd 18. However, preterm labor rate was statistically indistinguishable between the LPS group and low-dose LPS pretreatment group.

The number of litters, implantation sites per litter and resorptions per litter are presented in Table 1.

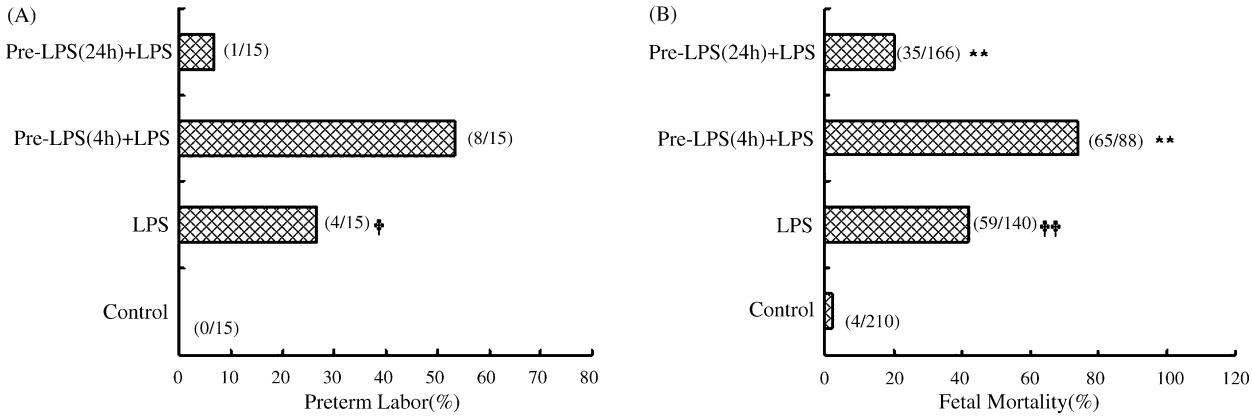


Fig. 1. The effects of low-dose LPS pretreatment on LPS-induced preterm labor and IUFD. All pregnant mice except controls received an intraperitoneal injection of high-dose LPS (120 μg/kg) on gd 15. Some pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 or 24 h before high-dose LPS (120 μg/kg). All pregnant mice were observed closedly for evidence of preterm labor. The pregnant mice who were not delivered were sacrificed on gd 18. The number of live fetuses and dead fetuses in each group are counted. (A) The effects of LPS on preterm labor. Numbers of litters in each group were presented in parentheses. (B) The effects of LPS on IUFD. The number of live fetuses and dead fetuses are presented in parentheses. Pre-LPS(4 h) + LPS, pretreatment with low-dose LPS (10 μg/kg) 4 h before high-dose LPS; pre-LPS(24 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 24 h before high-dose LPS. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with high-dose LPS group.

There were no differences in the number of implantation sites among different groups. Although there were fewer resorptions per litter in LPS alone group than those in the control, resorptions per litter were statistically indistinguishable among different groups. Preliminary results showed that administration of low-dose LPS (10 μg/kg, i.p.) to the pregnant mice on gd 14 or gd 15 did not increase fetal mortality (data not shown). As shown in Fig. 1B, administration of high-dose LPS (120 μg/kg, i.p.) to the pregnant mice on gd 15 resulted in 42.1% fetuses dead. Whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced developmental toxicity depends on the interval between the two doses of LPS. When administered 24 h before high-dose LPS, low-dose LPS pretreatment significantly decreased high-dose LPS-induced fetal mortality (42.1% versus 21.1%, $P < 0.01$). However, when administered 4 h before high-dose LPS, LPS hyporesponsiveness did not occur and low-dose LPS pretreatment in fact aggravated high-dose LPS-induced IUFD (73.9% versus 42.1%, $P < 0.01$).

### 3.2. Effects of low-dose LPS pretreatment on LPS-induced IUGR

The effects of maternally administered LPS on fetal crown-rump length and fetal weight were analyzed. The results showed that crown-rump length and fetal weight were statistically indistinguishable among different groups, although there was a trend for LPS-treated mice to have a shorter crown-rump length and a lighter fetal weight (see Fig. 2). Low-dose LPS pretreatment had no effect on crown-rump length and fetal weight.

### 3.3. Effects of low-dose LPS pretreatment on LPS-induced TNF-α releases

The effects of LPS on TNF-α in maternal serum and amniotic fluid are analyzed. As shown in Fig. 3, TNF-α in maternal serum and amniotic fluid was significantly increased at 1.5 h after high-dose LPS treatment. Whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced TNF-α

Table 1
Comparison of fetal outcomes among different groups

| Treatments | Litters | Implantation sites per litter ($\bar{x} \pm$ S.E.M.) | Resorptions per litter ($\bar{x} \pm$ S.E.M.) |
|---|---|---|---|
| Control | 15 | 14.9 ± 0.39 | 0.80 ± 0.27 |
| LPS (120 μg/kg) | 11 | 13.0 ± 1.05 | 0.30 ± 0.14 |
| LPS pretreatment (10 μg/kg, 4 h) + LPS | 7 | 13.1 ± 1.03 | 0.57 ± 0.37 |
| LPS pretreatment (10 μg/kg, 24 h)+LPS | 14 | 12.5 ± 0.48 | 0.64 ± 0.22 |

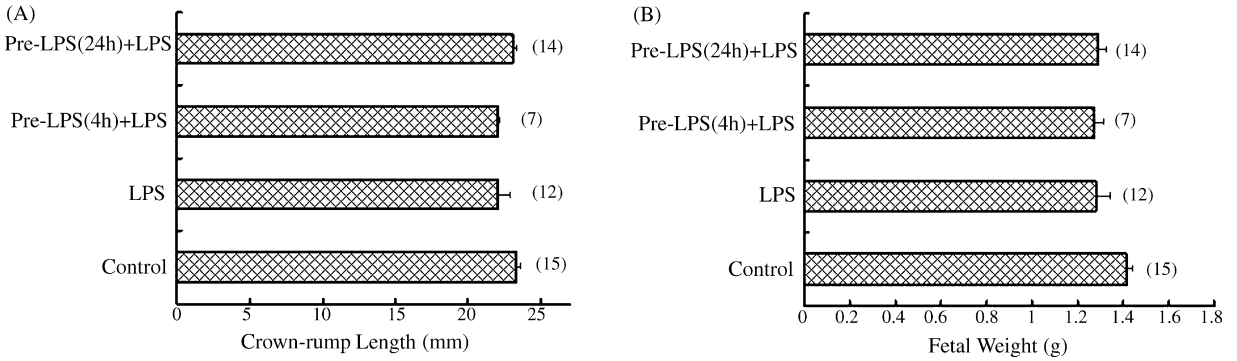


Fig. 2. The effects of low-dose LPS pretreatment on LPS-induced IUGR. All pregnant mice except controls received an intraperitoneal injection of high-dose LPS (120 μg/kg) on gd 15. Some pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 or 24 h before high-dose LPS (120 μg/kg). The pregnant mice were sacrificed on gd 18. Fetal weight and crown-rump length were measured. Numbers of litters in each group were presented in parentheses. (A) The effects of LPS on fetal crown-rump length. (B) The effects of LPS fetal weight. Pre-LPS(4 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 4 h before high-dose LPS; pre-LPS(24 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 24 h before high-dose LPS.

depends on the interval between the two doses of LPS. When administered 24 h before high-dose LPS, low-dose LPS pretreatment significantly attenuated subsequent high-dose LPS-evoked releases of TNF-α in maternal serum and amniotic fluid. However, when administered 4 h before high-dose LPS, low-dose LPS pretreatment had no effect on high-dose LPS-evoked releases of TNF-α in maternal serum and in fact aggravated high-dose LPS-evoked releases of TNF-α in amniotic fluid.

### *3.4. Effects of low-dose LPS pretreatment on LPS-induced IL-10 releases*

The effects of LPS on IL-10 are analyzed in maternal serum and amniotic fluid. As shown in Fig. 4, IL-10 in maternal serum and amniotic fluid was significantly increased at 1.5 h after high-dose LPS treatment. Surprisingly, whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced IL-10 also depends on the interval between

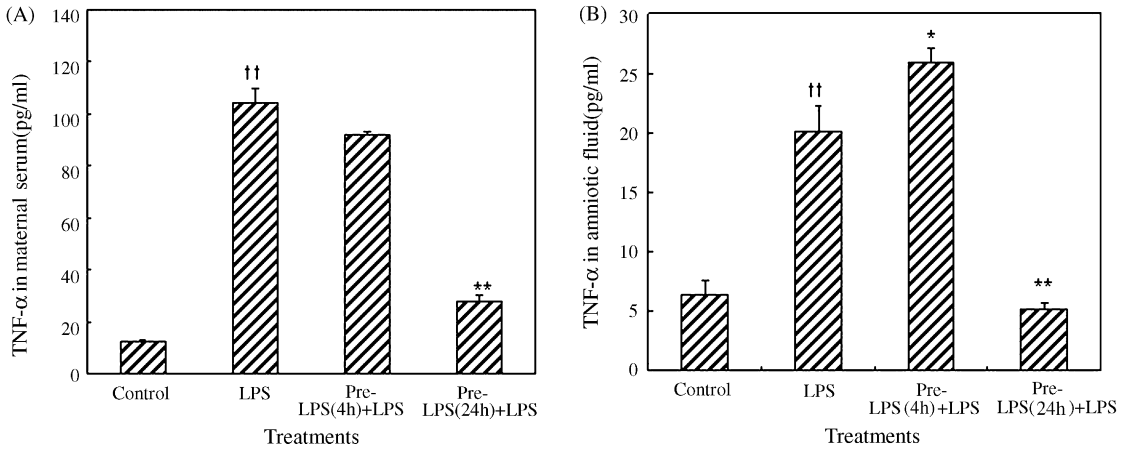


Fig. 3. The effects of low-dose LPS pretreatment on LPS-evoked TNF-α releases. All pregnant mice except controls received an intraperitoneal injection of high-dose LPS (120 μg/kg) on gd 15. Some pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 or 24 h before high-dose LPS (120 μg/kg, i.p.). The pregnant mice were sacrificed 1.5 h after high-dose LPS treatment. Maternal serum and amniotic fluid were collected for measurement of TNF-α level using ELISA. (A) The effects of LPS on TNF-α in maternal serum. (B) The effects of LPS on TNF-α in amniotic fluid. Data were expressed as means ± S.E.M. ($n = 12$). Pre-LPS(4 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 4 h before high-dose LPS; pre-LPS(24 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 24 h before high-dose LPS. $^{††}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with high-dose LPS group.

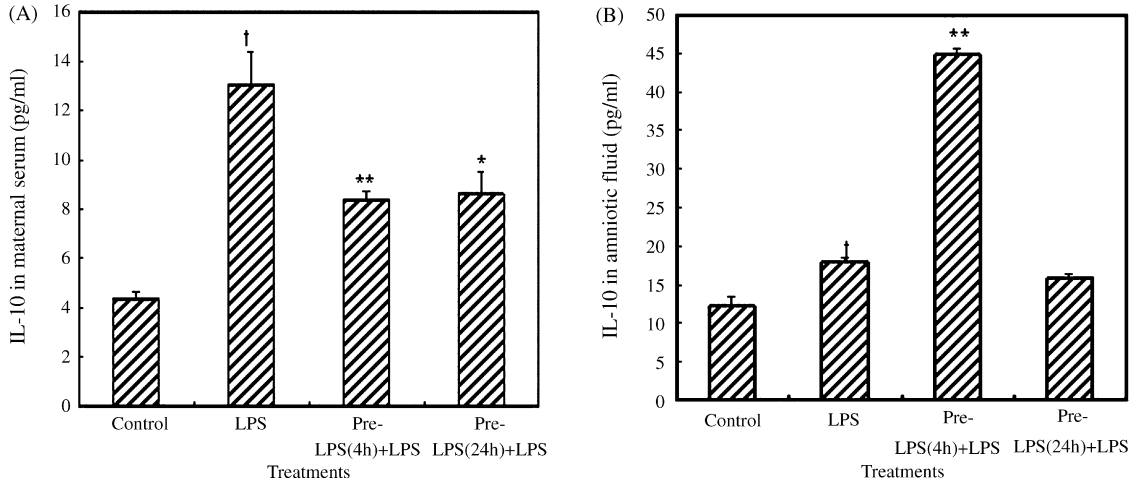


Fig. 4. The effects of low-dose LPS pretreatment on LPS-evoked IL-10 releases. All pregnant mice except controls received an intraperitoneal injection of high-dose LPS (120 μg/kg) on gd 15. Some pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 or 24 h before high-dose LPS (120 μg/kg, i.p.). The pregnant mice were sacrificed 1.5 h after high-dose LPS treatment. Maternal serum and amniotic fluid were collected for measurement of IL-10 level using ELISA. (A) The effects of LPS on IL-10 in maternal serum. (B) The effects of LPS on IL-10 in amniotic fluid. Data were expressed as means ± S.E.M. ($n = 12$). Pre-LPS(4 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 4 h before high-dose LPS; pre-LPS(24 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 24 h before high-dose LPS. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with high-dose LPS group.

the two doses of LPS. When administered 24 h before high-dose LPS, low-dose LPS pretreatment significantly inhibited high-dose LPS-evoked releases of IL-10 in maternal serum. By contrast, when administered 4 h before high-dose LPS, low-dose LPS pretreatment increased high-dose LPS-evoked releases of IL-10 in amniotic fluid.

### 3.4.1. *Effects of low-dose LPS pretreatment on LPS-induced oxidative stress in mouse placenta*

LPS-induced oxidative stress in mouse placenta was quantified by measuring placental GSH and TBARS. As expected, high-dose LPS significantly increased level of placental TBARS. By contrast, administration of high-dose LPS on gestational day 15 significantly decreased

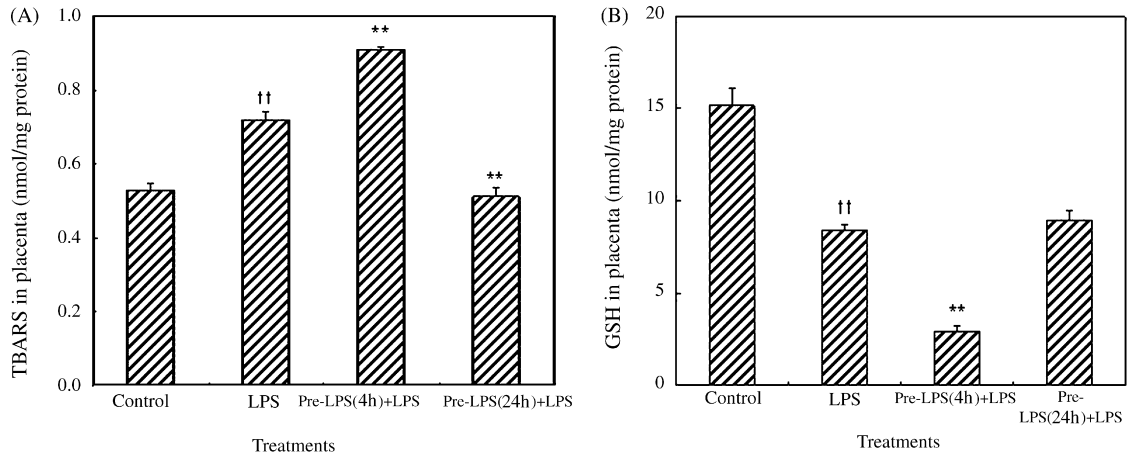


Fig. 5. The effects of low-dose LPS pretreatment on LPS-induced lipid peroxidation and GSH depletion in mouse placenta. All pregnant mice except controls received an intraperitoneal injection of high-dose LPS (120 μg/kg) on gd 15. Some pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 or 24 h before high-dose LPS (120 μg/kg, i.p.). The pregnant mice were sacrificed 6 h after high-dose LPS treatment. Placentas were excised for measurement of TBARS and GSH. (A) The effects of low-dose LPS pretreatment on LPS-induced lipid peroxidation. (B) The effects of low-dose LPS pretreatment on LPS-induced GSH depletion. Data were expressed as means ± S.E.M. of 12 mice in each point. Pre-LPS(4 h) + LPS, pretreatment with low-dose LPS (10 μg/kg) 4 h before high-dose LPS; pre-LPS(24 h) + LPS, pretreatment with low-dose LPS (10 μg/kg) 24 h before high-dose LPS. $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ vs. LPS-treated control.

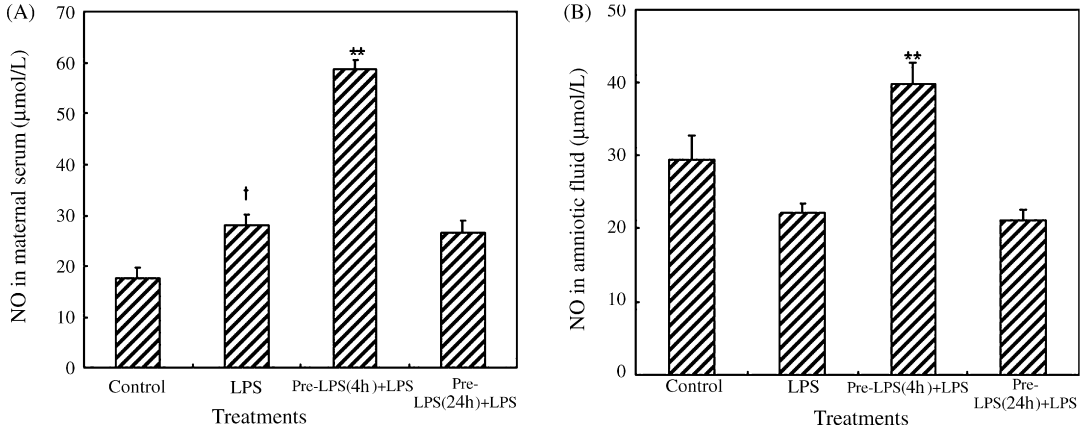


Fig. 6. The effects of low-dose LPS pretreatment on LPS-induced nitric oxide production. All pregnant mice except controls received an intraperitoneal injection of high-dose LPS (120 μg/kg) on gd 15. Some pregnant mice were pretreated with low-dose LPS (10 μg/kg, i.p.) 4 or 24 h before high-dose LPS (120 μg/kg, i.p.). The pregnant mice were sacrificed 6 h after high-dose LPS treatment. Maternal serum and amniotic fluid were collected for measurement of nitrate plus nitrite level. Data were expressed as means ± S.E.M. ($n = 12$). Pre-LPS(4 h) + LPS, pretreatment with low-dose LPS (10 μg/kg) 4 h before high-dose LPS; pre-LPS(24 h)+LPS, pretreatment with low-dose LPS (10 μg/kg) 24 h before high-dose LPS. $^{\dagger}P < 0.05$ as compared with control group. $^{**}P < 0.01$ as compared with LPS-treated group.

placental GSH. Whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced oxidative stress depends on the interval between the two doses of LPS. When administered 24 h before high-dose LPS, low-dose LPS pretreatment significantly counteracted high-dose LPS-induced placental lipid peroxidation. On the contrary, when administered 4 h before high-dose LPS, low-dose LPS pretreatment did not induce LPS tolerance and in fact aggravated high-dose LPS-induced lipid peroxidation and GSH depletion in mouse placenta (Fig. 5).

### *3.4.2. Effects of low-dose LPS pretreatment on LPS-induced nitric oxide production*

The stable end products of L-arginine-dependent nitric oxide synthesis, nitrate plus nitrite, are analyzed in maternal serum and amniotic fluid. As shown in Fig. 6, nitrate plus nitrite was slightly increased in maternal serum 6 h after a single dose of LPS (120 μg/kg, i.p.) was administered to the pregnant mice. However, a low-dose LPS pretreatment did not induce a reduced sensitivity to subsequent high-dose LPS-induced nitric oxide production. On the contrary, when administered 4 h before high-dose LPS, low-dose LPS pretreatment aggravated high-dose LPS-evoked increase in nitrate plus nitrite in maternal serum and amniotic fluid.

## 4. Discussion

First, we investigated the effects of high-dose LPS on intra-uterine fetal development. The pregnant mice received an intraperitoneal injection of LPS (120 μg/kg) on gd 15. As expected, preterm labor rate was dramatically increased in response to LPS challenge. In addition, administration of a single dose LPS (120 μg/kg, i.p.) resulted in 42.1% fetuses dead. Our earlier studies showed that administration of three doses LPS (75 μg/kg/day) from gd 15 to gd 17 greatly reduced fetal weight and crown-rump length (Xu et al., 2005, 2006a,b). However, the present study found that administration of a single dose LPS (120 μg/kg) on gd 15 did not statistically decreased crown-rump length and fetal weight, although there was a trend for LPS-treated mice to have a shorter crown-rump length and a lighter fetal weight.

LPS hyporesponsiveness has been observed in vivo febrile response and escape from lethality as well as in vitro with a reduced production of inflammatory cytokines in response to a secondary stimulation with LPS (Erroi et al., 1993; Medvedev et al., 2000). In the present study, we investigated whether a low-dose LPS exposure during pregnancy also produce a LPS hyporesponsiveness. Interestingly, whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced developmental toxicity depends on the interval between the two doses of LPS. When administered 24 h before high-dose LPS, low-dose LPS pretreatment obviously decreased subsequent high-dose LPS-induced intra-uterine fetal mortality. However, when administered 4 h before high-dose LPS, low-dose LPS did not induce LPS hyporesponsiveness and in fact aggravated high-dose LPS-induced IUFD.

Numerous studies have demonstrated that maternally administered LPS stimulates the production of a variety

of cytokines (Gayle et al., 2004), of which TNF-α is the major mediator leading to IUFD and preterm delivery (Leazer et al., 2002; Xu et al., 2006a,b). To investigate whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced TNF-α production, TNF-α was measured at 1.5 h after high-dose LPS treatment. In accordance with protective effect on IUFD, low-dose LPS pretreatment 24 h before high-dose LPS greatly reduced subsequent high-dose LPS-evoked releases of TNF-α in maternal serum and amniotic fluid. However, LPS tolerance did not occur when low-dose LPS was administered 4 h before high-dose LPS. These results suggest that the protective effect of low-dose LPS pretreatment on LPS-induced IUFD and preterm labor might, at least in part, be due to decreased TNF-α production in maternal serum and amniotic fluid.

IL-10 is an important antiinflammatory cytokine. An earlier study showed that IL-10 protected rodents against LPS-induced IUFD and IUGR (Rivera et al., 1998). A recent study found that IL-10 protects mice against LPS-induced preterm labor (Robertson et al., 2006). Therefore, it is especially interesting whether the protective effect of low-dose LPS pretreatment on LPS-induced developmental toxicity is due to increased IL-10 release. In the present study, we measured IL-10 at 1.5 h after high-dose LPS treatment. As expected, IL-10 was increased significantly in maternal serum and slightly in amniotic fluid. Surprisingly, when administered 24 h before high-dose LPS, low-dose LPS pretreatment significantly attenuated high-dose LPS-evoked releases of IL-10 in maternal serum. By contrast, when administered 4 h before high-dose LPS, low-dose LPS pretreatment greatly aggravated high-dose LPS-evoked releases of IL-10 in amniotic fluid.

LPS, a potent activator for macrophages, stimulates macrophages to generate reactive oxygen species. Several studies showed that LPS increased the levels of placental 4-hydroxy-2-nonenal (HNE)-modified proteins and TBARS, markers of oxidative stress (Ejima et al., 2000; Chen et al., 2005). Recently, we and others demonstrated that ROS contribute to LPS-induced IUFD, IUGR and preterm labor (Buhimschi et al., 2003; Xu et al., 2005, 2006a,b). To investigate whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced oxidative stress, we measured TBARS and GSH in mouse placenta. When administered 24 h before high-dose LPS, low-dose LPS pretreatment significantly protected against high-dose LPS-induced placental lipid peroxidation. On the contrary, when administered 4 h before high-dose LPS, low-dose LPS pretreatment did not induce LPS tolerance and in fact aggravated high-dose LPS-induced lipid peroxidation and GSH depletion in mouse placenta. These results could explain why low-dose LPS pretreatment 4 h before high-dose LPS aggravates high-dose LPS-induced IUFD.

Nitric oxide plays an important role in embryonic implantation, decidualization, vasodilatation and myometrial relaxation. However, a recent study found that high concentrations of nitric oxide contribute to LPS-induced embryonic resorption (Ogando et al., 2003). Aminoguanidine, an inhibitor of inducible nitric oxide synthase, reversed LPS-induced embryonic resorption and abortion (Athanassakis et al., 1999). To investigate whether a low-dose LPS pretreatment induce a reduced sensitivity to subsequent high-dose LPS-induced nitric oxide, nitrate plus nitrite, stable end products of L-arginine-dependent nitric oxide synthesis, was measured at 6 h after high-dose LPS treatment. As expected, nitrate plus nitrite was significantly increased in maternal serum. However, a low-dose LPS pretreatment did not induce a reduced sensitivity to subsequent high-dose LPS-induced nitric oxide production. On the contrary, when administered 4 h before high-dose LPS, low-dose LPS pretreatment aggravated high-dose LPS-evoked increase in nitrate plus nitrite in maternal serum and amniotic fluid. These results could also explain why low-dose LPS pretreatment 4 h before high-dose LPS aggravates high-dose LPS-induced IUFD.

In summary, the present study indicates whether a low-dose LPS exposure during pregnancy produce LPS hyporesponsiveness depends on the interval between the two doses of LPS. When administered 24 h before high-dose LPS, a low-dose LPS pretreatment induces a reduced sensitivity to subsequent high-dose LPS-induced IUFD, TNF-α in maternal serum and amniotic fluid and oxidative stress in placenta. When administered 4 h before high-dose LPS, low-dose LPS did not induce LPS hyporesponsiveness and in fact aggravated high-dose LPS-induced IUFD, oxidative stress in placenta and nitric oxide production in maternal serum and amniotic fluid.

## Acknowledgements

The project was supported by National Natural Science Foundation of China (30371667) and Anhui Provincial Natural Science Foundation (050430714).

## References

Athanassakis, I., Aifantis, I., Ranella, A., Giouremou, K., Vassiliadis, S., 1999. Inhibition of nitric oxide production rescues LPS-induced fetal abortion in mice. Nitric Oxide 3, 216–224.

Bell, M.J., Hallenbeck, J.M., Gallo, V., 2004. Determining the fetal inflammatory response in an experimental model of intrauterine inflammation in rats. Pediatr. Res. 56, 541–546.

Buhimschi, I.A., Buhimschi, C.S., Weiner, C.P., 2003. Protective effect of *N*-acetylcysteine against fetal fetal death and preterm labor induced by maternal inflammation. Am. J. Obstet. Gynecol. 188, 203–208.

Chen, Y.H., Wang, J.P., Wang, H., Sun, M.F., Wei, L.Z., Wei, W., Xu, D.X., 2005. Lipopolysaccharide treatment downregulates the expression of the pregnane X receptor, cyp3a11 and mdr1a genes in mouse placenta. Toxicology 211, 242–252.

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

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. Biol. Reprod. 62, 178–185.

Erroi, A., Fantuzzi, G., Mengozzi, M., Sironi, M., Orencole, S.F., Clark, B.D., Dinarello, C.A., Isetta, A., Gnocchi, P., Giovarelli, M., 1993. Differential regulation of cytokine production in lipopolysaccharide tolerance in mice. Infect. Immun. 61, 4356–4359.

Fan, J., Kapus, A., Marsden, P.A., Li, Y.H., Oreopoulos, G., Marshall, J.C., Frantz, S., Kelly, R.A., Medzhitov, R., Rotstein, O.D., 2002. Regulation of toll-Like receptor 4 expression in the lung following hemorrhagic shock and lipopolysaccharide. J. Immunol. 168, 5252–5259.

Fukui, H., Brauner, B., Bode, J.C., Bode, C., 1991. Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: reevaluation with an improved chromogenic assay. J. Hepatol. 12, 162–169.

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. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R1024–R1029.

Gendron, R.L., Nestel, F.P., Lapp, W.S., Baines, M.G., 1990. Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor-alpha. J. Reprod. Fertil. 90, 395–402.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212.

Grisham, M.B., Johnson, G.G., Lancaster Jr., J.R., 1996. Quantitation of nitrate and nitrite in extracellular fluids. Methods Enzymol. 268, 237–246.

Gross, G., Imamura, T., Vogt, S.K., Wozniak, D.F., Nelson, D.M., Sadovsky, Y., Muglia, L.J., 2000. Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278, R1415–R1423.

Jacob, A.L., Goldberg, P.K., Bloom, N., Degenshein, G.A., Kozinn, P.J., 1997. Endotoxin and bacteria in portal blood. Gastroenterology 72, 1268–1270.

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. Reprod. Toxicol. 16, 173–179.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Medvedev, A.E., Kopydlowski, K.M., Vogel, S.N., 2000. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. J. Immunol. 164, 5564–5574.

Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., Akira, S., 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. J. Immunol. 164, 3476–3479.

Ogando, D.G., Paz, D., Cella, M., Franchi, A.M., 2003. The functional role of increased production of nitric oxide in lipopolysaccharide-induced embryonic resorption in mice. Reproduction 125, 95–110.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 44, 276–278.

Rivera, D.L., Olister, S.M., Liu, X., Thompson, J.H., Zhang, X.J., Pennline, K., Azuero, R., Clark, D.A., Miller, M.J., 1998. Interleukin-10 attenuates experimental fetal growth restriction and demise. FASEB J. 12, 189–197.

Robertson, S.A., Skinner, R.J., Care, A.S., 2006. Essential role for IL-10 in resistance to lipopolysaccharide-induced preterm labor in mice. J. Immunol. 177, 4888–4896.

Romero, R., Roslansky, P., Oyarzun, E., Wan, M., Emamian, M., Novitsky, T.J., Gould, M.J., Hobbins, J.C., 1988. Labor and infection. II. Bacterial endotoxin in amniotic fluid and its relationship to the onset of preterm labor. Am. J. Obstet. Gynecol. 158, 1044–1049.

Sakai, M., Tanebe, K., Sasaki, Y., Momma, K., Yoneda, S., Saito, S., 2001. Evaluation of the tocolytic effect of a selective cyclooxygenase-2 inhibitor in a mouse model of lipopolysaccharide-induced preterm delivery. Mol. Hum. Reprod. 7, 595–602.

Silver, R.M., Lohner, W.S., Daynes, R.A., Mitchell, M.D., Branch, D.W., 1994. Lipopolysaccharide-induced fetal death: the role of tumor-necrosis factor alpha. Biol. Reprod. 50, 1108–1112.

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. J. Clin. Invest. 95, 725–731.

Vizi, E.S., Szelenyi, J., Selmeczy, Z.S., Papp, Z., Nemeth, Z.H., Hasko, G., 2001. Enhanced tumor necrosis factor-alpha-specific and decreased interleukin-10-specific immune responses to LPS during the third trimester of pregnancy in mice. J. Endocrinol. 171, 355–361.

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 intra-uterine growth retardation in mice. Toxicol. Sci. 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 intra-uterine fetal growth restriction and skeletal development retardation in mice. Toxicol. Lett. 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. Am. J. Obstet. Gynecol. 195, 1707–1714.