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Tumor necrosis factor alpha partially contributes to lipopolysaccharide-induced intra-uterine fetal growth restriction and skeletal development retardation in mice

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Abstract

Maternal infection is a cause of adverse developmental outcomes. Lipopolysaccharide (LPS)-induced embryonic resorption, intra-uterine fetal death (IUFD) and preterm labor have been well characterized. In the present study, we investigated the effects of maternal LPS exposure on intra-uterine fetal growth and skeletal development. All pregnant mice except controls received an intraperitoneal injection of LPS (75 μg/kg) on gestational days (GD) 15–17. The number of live fetuses, dead fetuses and resorption sites was counted on GD 18. Live fetuses in each litter were weighed. Crown-rump and tail lengths were examined and skeletal development was evaluated. As expected, perinatal LPS exposure resulted in 63.2% fetal death. LPS significantly lowered fetal weight, reduced crown-rump and tail lengths, and retarded skeletal ossification in caudal vertebrae, anterior and posterior phalanges, and supraoccipital bone. Additional experiment showed that a single dose of LPS (75 μg/kg, i.p.) on GD 15 increased the expression of TNF-α mRNA in maternal liver and placenta and TNF-α concentration in maternal serum and amniotic fluid. Furthermore, pentoxifylline, an inhibitor of TNF-α synthesis, significantly inhibited TNF-α production, reduced fetal mortality, and reversed LPS-induced fetal intra-uterine growth restriction and skeletal development retardation. Taken together, these results suggest that TNF-α is, at least in part, involved in LPS-induced intra-uterine fetal death, intra-uterine growth restriction and skeletal development retardation.

Keywords: Tumor necrosis factor alpha; Pentoxifylline; Lipopolysaccharide; Intra-uterine growth restriction; Skeletal development retardation

Abbreviations: GSH, glutathione; iNOS, inducible nitric oxide synthase; IUFD, intra-uterine fetal death; IUGR, intra-uterine growth restriction; LPS, lipopolysaccharide; NO, nitric oxide; $O_2^{\bullet -}$, superoxide anion; PTX, pentoxifylline; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substance; TNF-α, tumor necrosis factor alpha

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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., 1977). 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). LPS has been associated with adverse developmental outcome, including embryonic resorption, intra-uterine fetal death (IUFD), intra-uterine growth restriction (IUGR) and preterm labor and delivery in animals (O'Sullivan et al., 1988; Collins et al., 1994). However, the exact mechanism of LPS-induced developmental toxicity remains unclear.

Eicosanoids have been demonstrated to be important mediators of LPS-induced IUFD and preterm labor. Several studies indicate that LPS-induced embryo death was associated with COX-2-mediated eicosanoid production. Silver et al. (1995) reported that pregnant C3H/HeN mice injected with LPS showed an increase in decidual eicosanoid production and COX_2 expression, followed by a dose-dependent increase in embryo death. Moreover, COX_2 pretreatment with suppressors, indomethacin, SC236 and Celecoxib, significantly decreased LPS-induced the incidence of fetal death and prevented LPS-induced preterm delivery (Gross et al., 2000; Sakai et al., 2001). On the other hand, nitric oxide (NO) has been associated with LPS-induced embryonic resorption and abortion. A recent study showed that maternal LPS exposure at early gestational stage significantly increased inducible nitric oxide synthase (iNOS) expression in decidual and myometrial cells and nitric oxide production in decidual and uterine (Ogando et al., 2003). Aminoguanidine, a specific inhibitor of iNOS activity, reversed LPS-induced embryonic resorption and abortion (Athanassakis et al., 1999).

Numerous studies showed that maternal LPS exposure increased TNF-α production in maternal serum and amniotic fluid (Bell et al., 2004; Vizi et al., 2001; Gayle et al., 2004). TNF-α has been associated with LPS-induced intra-uterine fetal death and preterm labor (Gendron et al., 1990; Silver et al., 1994; Leazer et al., 2002). The purpose of the present study is to investigate the effects of maternal LPS exposure on intra-uterine fetal growth and skeletal development, and assess the role of TNF-α on LPS-induced intra-uterine fetal growth restriction (IUGR) and skeletal development retardation in mice.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and pentoxifylline (PTX) were 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: 30–32 g; female mice: 26–28 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 included two separate experiments.

2.2.1. Experiment 1

The timed pregnant mice were divided into four groups randomly. All pregnant mice except controls received an intraperitoneal injection of LPS (75 μg/kg) on GD 15–17. In PTX + LPS group, the pregnant mice were injected with PTX (100 mg/kg, i.p.) at 30 min before LPS. The saline- and PTX-treated pregnant mice served as controls. All 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 was counted. Live fetuses in each litter were weighed. Crown-rump and tail lengths were measured. All fetuses were then stored in ethanol a minimum of 2 weeks for subsequent skeletal evaluation.

2.2.2. Experiment 2

The timed pregnant mice were divided into four groups randomly. All pregnant mice except controls received an intraperitoneal injection of LPS (75 μg/kg) on GD 15. In PTX + LPS group, the pregnant mice were injected with PTX (100 mg/kg, i.p.) at 30 min before LPS. The saline- and PTX-treated pregnant mice served as controls. Half of the dams were sacrificed at 1.5 h after LPS treatment. Maternal liver and placenta were dissected for total RNA extraction. Maternal serum and amniotic fluid were collected for measurement of TNF-α concentration. The remaining dams were sacrificed at 6 h after LPS treatment. Maternal liver, placenta and fetal liver samples were excised for measurement of TBARS and GSH contents.

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 stored in ethanol were cleared from skin, viscera and adipose tissue. Fetuses were then incubated in acetone overnight and subsequently macerated and stained with alizarin red S for 2 days. After an overnight incubation in 70% ethanol/glycerol/benzyl alcohol, the fetuses were stored in glycerol until examination. Skeletal evaluation included determination of the degree of ossification of the phalanges, metacarpals, vertebrae, sternatrae and skull. The size of ossification of the supraoccipital was scored (1 = well ossified, 4 = completely unossified).

2.4. Isolation of total RNA and RT

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNase-free DNase (Promega) was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. Total RNA was stored at −80 °C. For the synthesis of cDNA, 2.0 μg of total RNA from each sample was resuspended in a 20-μl final volume of reaction buffer, which contained 25 mM Tris–HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each dNTP and 0.5 mg oligo$(dT)_{15}$ primer (Promega). After the reaction mixture reached 38 °C, 400 units of RT (Promega) was added to each tube and the sample was incubated for 60 min at 38 °C. Reverse transcription was stopped by denaturing the enzyme at 95 °C.

2.5. PCR amplification

The final PCR mixture contained 2.5 μl of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP mixture, 1 U of Taq DNA polymerase, 1 μM sense and antisense primers, and sterile water to 50 μl. The reaction mixture was covered with mineral oil. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on each individual sample as an internal positive-control standard. Following primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China), according to sequence designs previously described (Xu et al., 2004).

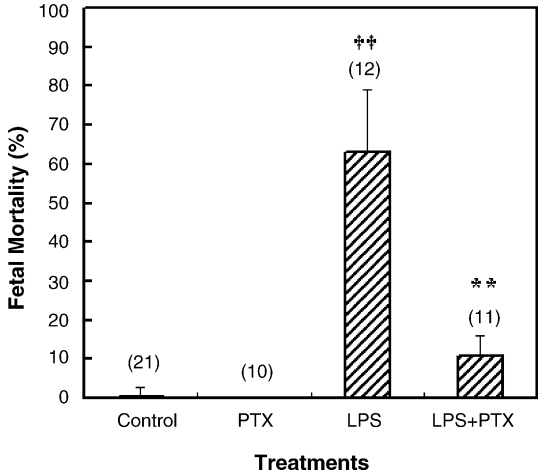

Fig. 1. LPS-induced IUFD. All pregnant mice except controls were injected with LPS (75 μg/kg, i.p.) on GD 15–17. In LPS + PTX group, the pregnant mice were injected with 100 mg/kg of PTX (i.p.) at 30 min before LPS treatment. The saline- and PTX-treated pregnant mice served as controls. The pregnant mice were sacrificed on GD 18. The number of live fetuses and dead fetuses were counted. Numbers of litters in each group were presented in parentheses. $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with LPS-treated group.

GAPDH, 5′-GAG GGG CCA TCC ACA GTC TTC-3′ and 5′-CAT CAC CAT CTT CCA GGA GCG-3′; TNF-α, 5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3′ and 5′-ACA TTC GAG GCT CCA GTG AAT TCG G-3′. The sizes of amplified PCR products were 340 bp for *GAPDH* and 307 bp for TNF-α. Number of cycles and annealing temperature were optimized for each primer pair. For *GAPDH*, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min. For TNF-α and IL-1β, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 30 cycles each of denaturation at 94 °C for 45 s, annealing of primer and fragment at 60 °C for 45 s, and primer extension at 72 °C for 1 min. A final extension of 72 °C for 10 min was included. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma) for 45 min. The pBR322 DNA digested with Alu I was used for molecular markers (MBI

Table 1
Comparison of fetal outcomes among different groups

Treatments	Litters	Implantation sites per litter ($\bar{X} \pm S$)	Resorptions per litter ($\bar{X} \pm S$)	Live fetuses per litter ($\bar{X} \pm S$)	Dead fetuses per litter ($\bar{X} \pm S$)
Control	21	11.6 ± 2.02	0.52 ± 0.99	11.00 ± 2.15	0.05 ± 1.09
PTX (100 mg/kg)	10	12.1 ± 1.87	0.60 ± 0.92	11.50 ± 2.06	0
LPS (75 μg/kg)	12	13.4 ± 1.98	0.50 ± 0.86	4.75 ± 2.25[a]	8.17 ± 1.95[a]
LPS (75 μg/kg) + PTX (100 mg/kg)	11	12.9 ± 1.90	1.00 ± 1.75	10.64 ± 2.12[b]	1.27 ± 1.12[b]

[a] Significantly different from control, $P < 0.01$.
[b] Significantly different from LPS group, $P < 0.01$.

Fermentas). Agarose gels were stained with 0.5 mg/ml ethidium bromide (Sigma) TBE buffer.

2.6. Measurement for TNF-α concentration

Mouse TNF-α concentration in maternal serum and amniotic fluid was measured by enzyme-linked immunosorbent assay (R&D, Minneapolis, MN), following the manufacturer's instructions.

2.7. Determination of glutathione content

The glutathione (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).

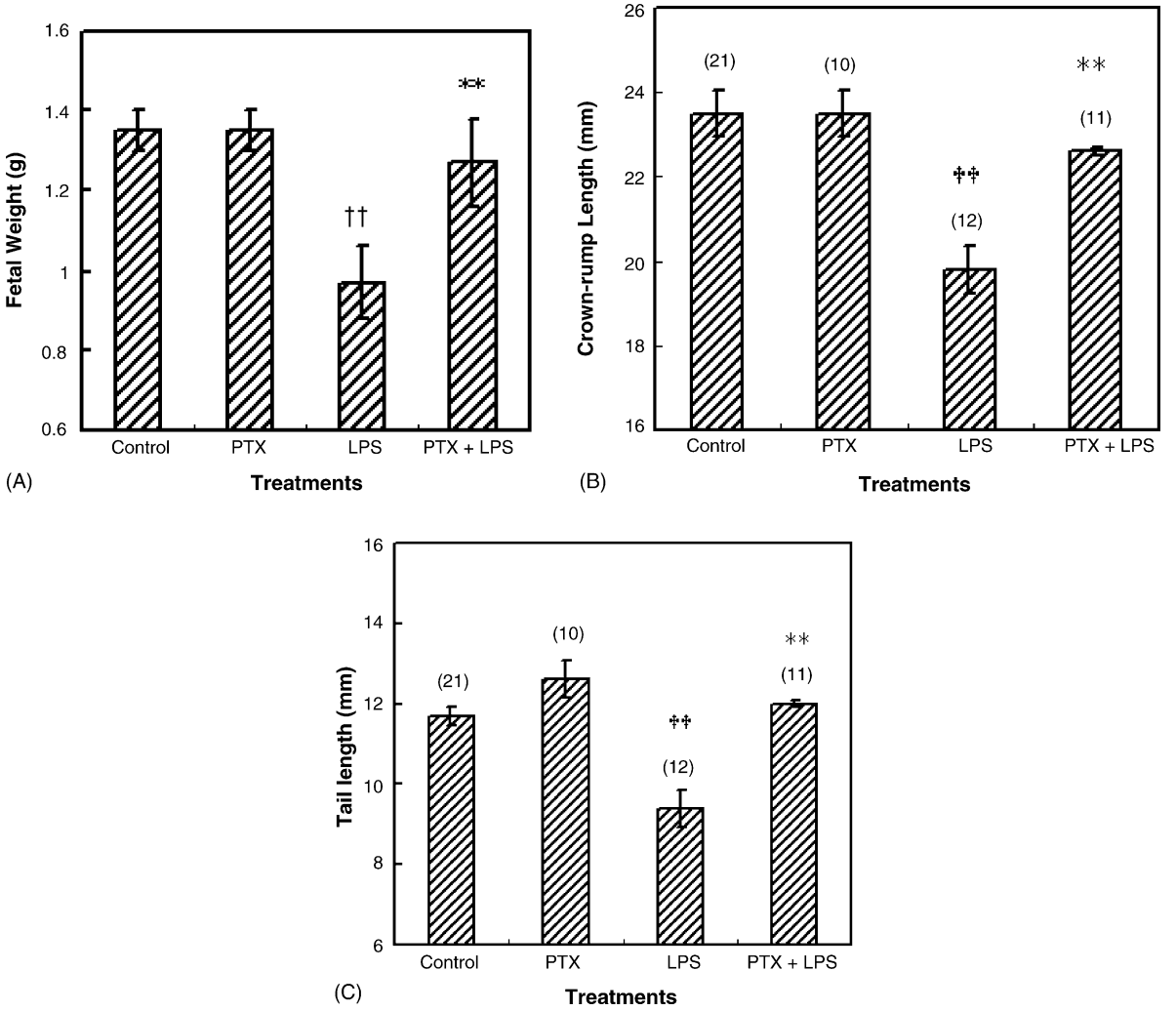

Fig. 2. LPS-induced IUGR. All pregnant mice except controls were injected with LPS (75 μg/kg, i.p.) on GD 15–17. In LPS + PTX group, the pregnant mice were injected with 100 mg/kg of PTX (i.p.) at 30 min before LPS treatment. The saline- and PTX-treated pregnant mice served as controls. The pregnant mice were sacrificed on GD 18. Fetal weight and crown-rump and tail lengths were measured. Numbers of litters in each group were presented in parentheses. (A) The effects of LPS on fetal weight. (B) The effects of LPS on fetal crown-rump length. (C) The effects of LPS on fetal tail lengths. $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with LPS-treated group.

2.8. Lipid peroxidation assay

Lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (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.

2.9. Statistical analysis

The litter was considered the unit for statistical comparison among different groups. Fetal mortality was calculated per litter and then averaged per group. For fetal weight, crown-rump and tail lengths, and skeletal evaluation, 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 between the treated animals and the control and statistical significance.

3. Results

3.1. LPS-induced IUFD

The number of litters, implantation sites per litter, resorptions per litter, live fetuses per litter and dead fetuses per litter are presented in Table 1. Result showed that there were no differences in the number of implantation sites and resorptions per litter among different groups. LPS exhibited no obvious maternal side effects (data not shown). LPS-induced IUFD is presented in Fig. 1. Results showed that maternal LPS exposure on GD 15–17 resulted in 63.2% fetal death. PTX (100 mg/kg) alone did not cause fetal death. Pretreatment with PTX markedly decreased LPS-induced fetal mortality (63.2% versus 10.7%, $P < 0.01$).

3.2. LPS-induced IUGR

LPS-induced IUGR is presented in Fig. 2. Results showed that maternal LPS exposure on GD 15–17 markedly reduced fetal weights and crown-rump and tail lengths. PTX (100 mg/kg) alone did not affect fetal weights and crown-rump and tail lengths. Pretreatment

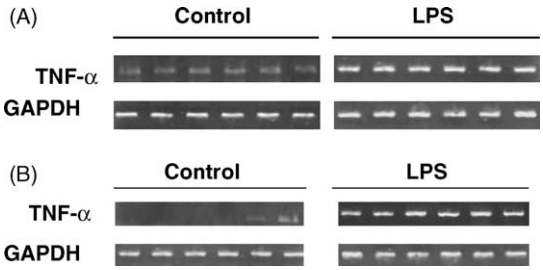

Fig. 3. The effects of LPS on TNF-α mRNA expression. All pregnant mice except controls were injected with LPS (75 μg/kg, i.p.) on GD 15. The saline-treated pregnant mice served as controls. Maternal liver and placenta were excised at 1.5 h after LPS for total RNA extraction. TNF-α mRNA levels were determined using RT-PCR. (A) The effects of LPS on TNF-α mRNA levels in maternal liver. (B) The effects of LPS on TNF-α mRNA levels in placenta.

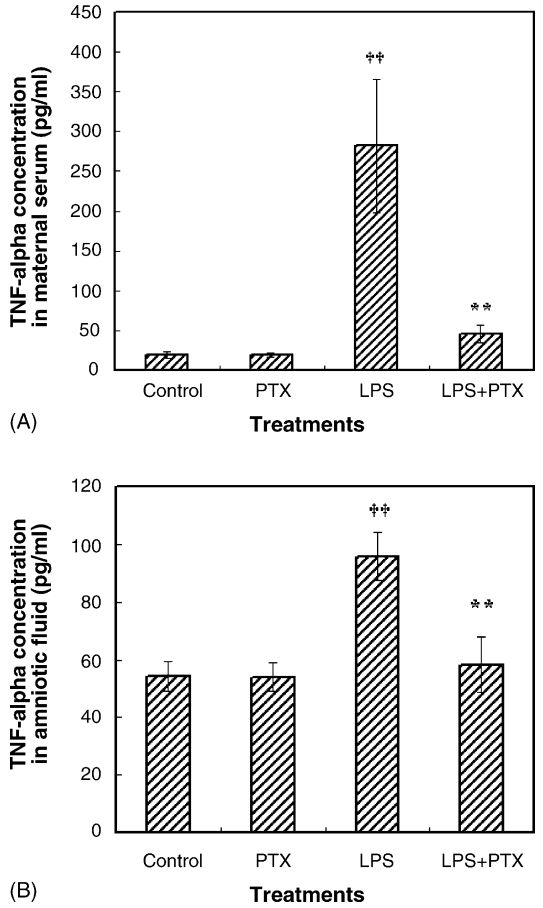

Fig. 4. The effects of LPS on TNF-α production. All pregnant mice except controls were injected with LPS (75 μg/kg, i.p.) on GD 15. In LPS + PTX group, the pregnant mice were injected with 100 mg/kg of PTX (i.p.) at 30 min before LPS. The saline- and PTX-treated pregnant mice served as controls. TNF-α level in maternal serum and amniotic fluid was measured using ELISA. Data were expressed as means ± S.E.M. of 12 mice in each point. (A) TNF-α level in maternal serum. (B) TNF-α level in amniotic fluid. $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with LPS group.

Table 2
The effects of PTX on LPS-induced fetal skeletal development retardation

	Control ($n=21$)	PTX ($n=10$)	LPS ($n=12$)	PTX + LPS ($n=11$)
No. examined				
Fetuses	231	115	57	117
Scores				
Supraoccipital bone[a]	1.02 ± 0.01	1.02 ± 0.01	$2.15 \pm 0.25^{\dagger\dagger}$	$1.20 \pm 0.12^{*}$
Number ossified				
Sternum	6.00 ± 0.00	6.00 ± 0.00	5.87 ± 0.26	5.96 ± 0.08
Metacarpus	4.00 ± 0.00	4.00 ± 0.00	3.88 ± 0.29	3.99 ± 0.03
Anterior phalanx	4.00 ± 0.00	4.00 ± 0.00	$3.08 \pm 1.42^{\dagger}$	$3.88 \pm 0.20^{*}$
Metatarsus	4.98 ± 0.01	4.98 ± 0.01	4.56 ± 0.39	4.88 ± 0.16
Posterior phalanx	4.95 ± 0.02	4.95 ± 0.02	$2.99 \pm 2.09^{\dagger\dagger}$	$4.40 \pm 0.62^{*}$
Caudal vertebrae	6.30 ± 0.21	6.30 ± 0.21	$3.67 \pm 0.91^{\dagger\dagger}$	$5.08 \pm 1.25^{**}$

Note: Numbers of litters in each group were presented in parentheses. Significantly different from control $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$. Significantly different from LPS75 group $^{*}P<0.05$, $^{**}P<0.01$.

[a] Supraoccipital bone scores: 1 = well ossified, 4 = completely unossified.

with PTX returned fetal weight and crown-rump and tail lengths to control levels. Placental size was statistically indistinguishable among different groups (data not shown).

3.3. LPS-induced skeletal development retardation

The effects of LPS on fetal skeletal development are presented in Table 2. The fetal skeleton of the LPS-treated mice, compared with the control group, exhibited fewer ossification centers in caudal vertebrae, anterior and posterior phalanges. In addition, LPS also retarded supraoccipital ossification. PTX (100 mg/kg) alone did not affect skeletal development. Pretreatment with PTX restored the number of ossification centers to control levels.

3.4. LPS-induced TNF-α production

The effects of LPS on TNF-α mRNA expression in maternal liver and placenta are presented in Fig. 3. Results showed that a single dose of LPS (75 μg/kg) significantly increased TNF-α mRNA level in maternal liver and placenta. The effects of LPS on TNF-α level in maternal serum and amniotic fluid are presented in Fig. 4. Results showed that a single dose of LPS (75 μg/kg) markedly elevated TNF-α concentration in maternal serum and amniotic fluid. Pretreatment with PTX returned TNF-α concentration to control level.

3.5. LPS-induced lipid peroxidation

Lipid peroxidation was quantified by measuring TBARS. The effects of LPS on TBARS content were analyzed. As shown in Fig. 5, a single dose of LPS caused an increase in TBARS contents by about 90.1% in maternal liver, 87.5% in placenta and 78.3% in fetal liver. Pretreatment with PTX significantly attenuated LPS-induced increase in TBARS contents in maternal liver and placenta. However, PTX had less effect on LPS-induced increase in TBARS contents in fetal liver.

3.6. LPS-induced GSH depletion

The effects of LPS on GSH content are presented in Fig. 6. Results showed that maternal LPS exposure decreased GSH content by about 34.6% in maternal liver, 40.3% in placenta and 50.6% in fetal liver. Pretreatment with PTX did not attenuate LPS-induced GSH depletion in maternal liver and fetal liver. Interestingly, PTX aggravated LPS-induced GSH depletion in placenta.

4. Discussion

LPS has been associated with adverse developmental outcome, such as embryonic resorption, intra-uterine fetal death and preterm labor (O'Sullivan et al., 1988; Collins et al., 1994). Our earlier study found that maternal LPS exposure on GD 17 significantly downregulated the expressions of pregnane X receptor and cyp3a11 in fetal mouse liver (Xu et al., 2005). In the present study, we investigated the effects of maternal LPS exposure at late gestational stage on intra-uterine fetal growth and skeletal development in mice. The timed pregnant mice were intraperitoneally injected with LPS (75 μg/kg) daily on GD 15–17. As expected, maternal LPS exposure resulted in up to 63.2% fetal death. Furthermore, LPS dramatically reduced fetal weight and crown-rump and

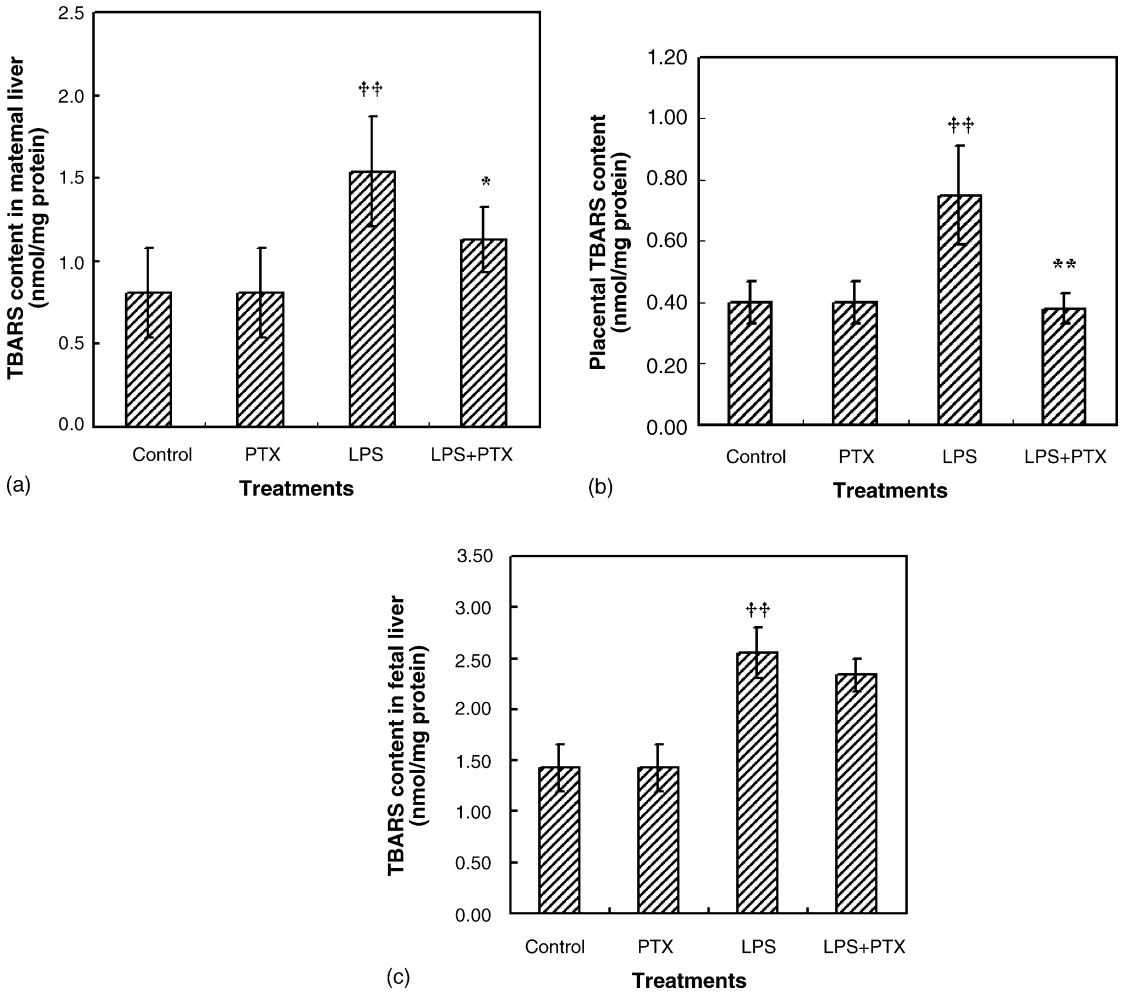

Fig. 5. The effects of LPS on lipid peroxidation. All pregnant mice except controls were injected with LPS (75 μg/kg, i.p.) on GD 15. In LPS + PTX group, the pregnant mice were injected with 100 mg/kg of PTX (i.p.) at 30 min before LPS treatment. The saline- and PTX-treated pregnant mice served as controls. TBARS level was analyzed as described in Section 2. (A) The effects of LPS on TBARS level in maternal liver. (B) The effects of LPS on TBARS level in placenta. (C) The effects of LPS on TBARS level in fetal liver. Data were expressed as means ± S.E.M. of 12 mice in each point. $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with LPS group.

tail lengths. These results are in agreement with earlier work of Rivera et al. (1998), in which administration with LPS (100 μg/kg) to rats on GD 14–20 caused 43% fetal death and reduced the size of the surviving fetuses. The present study also investigated the effects of maternal LPS exposure at late gestational stage on skeletal development. Result indicated that maternal LPS exposure on GD 15–17 significantly retarded skeletal ossification in caudal vertebrae, anterior and posterior phalanges, and supraoccipital bone.

Numerous studies indicated that LPS exposure increased TNF-α concentration in maternal serum and amniotic fluid (Silver et al., 1994; Leazer et al., 2002; Bell et al., 2004; Vizi et al., 2001; Gayle et al., 2004). A recent study showed that LPS upregulated TNF-α expression in glial cells in the mouse brain (Cheng et al., 2004). Moreover, LPS-activated microglia-derived cytokines, TNF-α and IL-18, may either inhibit the neuronal differentiation or induce neuronal cell death in the embryonic neural progenitor culture (Liu et al., 2005). In the present study, we investigated the effects of maternal LPS exposure on TNF-α production. Our results found that a single dose of LPS (75 μg/kg) on GD 15 significantly increased the expression of TNF-α mRNA in maternal liver and placenta and TNF-α concentration in maternal serum and amniotic fluid. Previous studies have demonstrated that TNF-α is involved in LPS-induced IUFD and preterm labor (Gendron et al., 1990; Silver et al., 1994; Leazer et al., 2002). To investigate the role of TNF-α on LPS-induced intra-uterine fetal growth

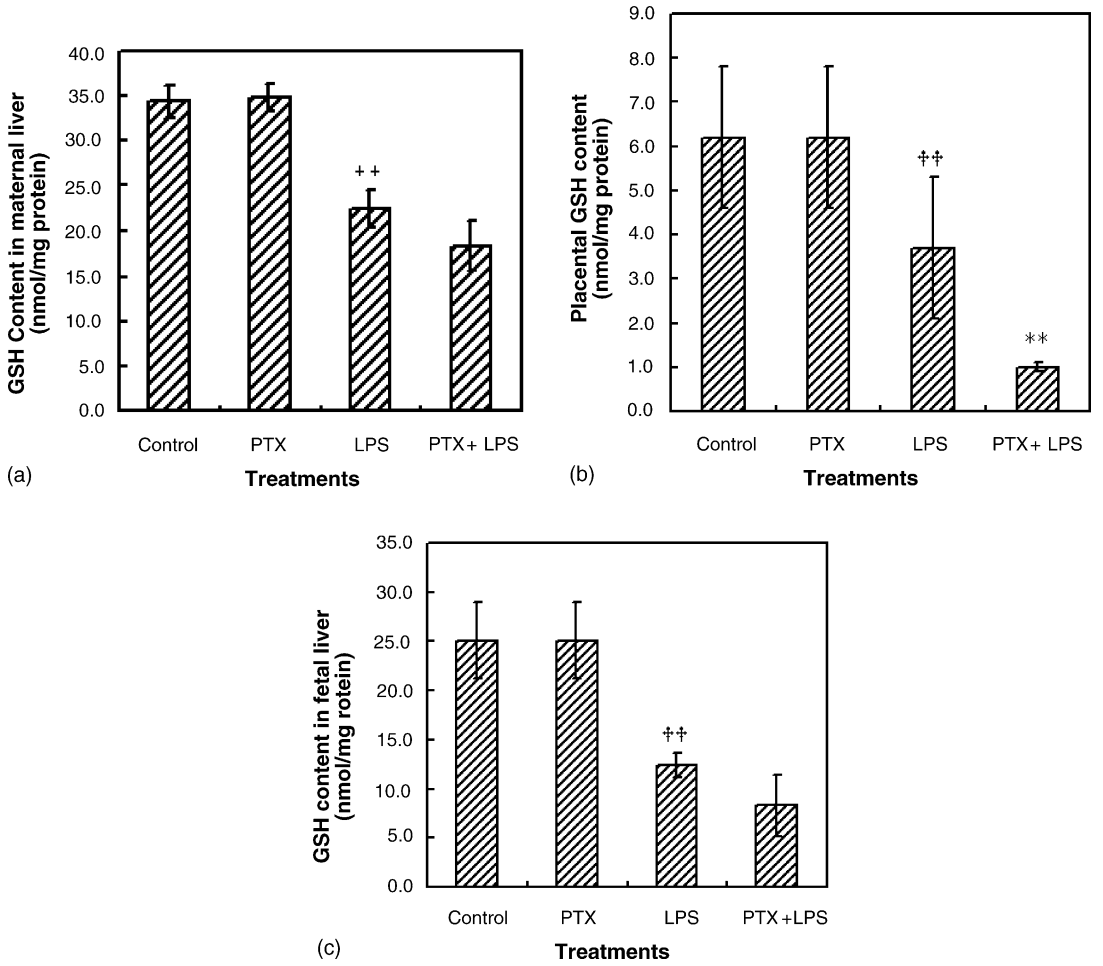

Fig. 6. The effects of LPS on GSH content. All pregnant mice except controls were injected with LPS (75 μg/kg, i.p.) on GD 15. In LPS + PTX group, the pregnant mice were injected with 100 mg/kg of PTX (i.p.) at 30 min before LPS treatment. The saline- and PTX-treated pregnant mice served as controls. GSH content was analyzed as described in Section 2. (A) The effects of PTX on GSH content in maternal liver. (B) The effects of PTX on GSH content in placenta. (C) The effects of PTX on GSH content in fetal liver. Data were expressed as means ± S.E.M. ($n = 12$). $^{\dagger\dagger}P < 0.01$ as compared with control group. $^{**}P < 0.01$ as compared with LPS group.

restriction and skeletal development retardation in mice, pentoxifylline (PTX), an inhibitor of TNF-α synthesis, was used to inhibit LPS-evoked TNF-α production. As expected, pretreatment with PTX significantly blocked LPS-induced increase in TNF-α concentration in maternal serum and amniotic fluid. Correspondingly, pretreatment with PTX markedly decreased fetal mortality and attenuated LPS-induced decrease in fetal weight and crown-rump and tail lengths. Interestingly, PTX also reversed LPS-induced skeletal ossification retardation in caudal vertebrae, anterior and posterior phalanges, and supraoccipital bone. These results suggest that TNF-α is involved in LPS-induced IUFD, IUGR and skeletal development retardation.

A recent study found that LPS-induced intra-uterine fetal death was not blocked by treatment with anti-TNF antibody that inhibited LPS-induced TNF-α production in pregnant females (Kohmura et al., 2000). Another important study by Casado et al. (1997), comparing difference in plasma TNF-α levels between in mother and in fetus, showed that less than 7% TNF-α was detected in the corresponding fetal serum although serum from mother treated with LPS exhibited a significant increase in TNF-α levels, suggesting a restricted permeability of placenta to proinflammatory cytokines. These results indicate that LPS-induced developmental toxicity can't be attributed to inhibition of TNF-α alone.

Indeed, LPS produces multiple pathophysiological effects. Several studies indicated that LPS stimulates macrophages to generate reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), and increases nitrotyrosine, a marker for $O_2^{\bullet -}$ and $ONOO^-$ formation, in

macrophage-rich organs (Bautista et al., 1990; Wheeler et al., 2001). Elevated levels of $O_2^{\bullet -}$ in macrophages are responsible for activation of NF-kB and releases of pro-inflammatory cytokines (Sass et al., 2001; Wheeler et al., 2001). The present study found that perinatal LPS exposure significantly increased thiobarbituric acid-reactive substance, a marker for lipid peroxidation, in maternal liver, placenta and fetal liver. In addition, a single dose of LPS significantly decreased GSH level in maternal liver, placenta and fetal liver. On the other hand, PTX has multiple pharmacological effects. First, PTX is an inducible nitric oxide synthase (iNOS) inhibitor (Loftis et al., 1997; Beshay et al., 2001). Next, PTX suppressed the activation of macrophage (such as Kupffer cells) (Kozaki et al., 1995) and attenuated LPS-evoked ROS production and oxidative stress (Hecht et al., 1995; Arias-Diaz et al., 1997). In this study, we found that pretreatment with PTX significantly alleviated LPS-induced lipid peroxidation in maternal liver and placenta. Therefore, the present study did not exclude that other mechanisms, such as ROS and NO, might also be involved in LPS-induced intra-uterine fetal growth restriction and skeletal development retardation.

In summary, the present results allow us to reach the following conclusions. Perinatal LPS exposure significantly elevated fetal mortality, reduced fetal weight and crown-rump and tail lengths, and retarded skeletal ossification in caudal vertebrae, anterior and posterior phalanges, and supraoccipital bone. TNF-α production is, at least in part, involved in LPS-induced intra-uterine fetal death and growth and skeletal development retardation.

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