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Melatonin protects against lipopolysaccharide-induced intra-uterine fetal death and growth retardation in mice

Abstract: Lipopolysaccharide (LPS) has been associated with adverse developmental outcomes, including intra-uterine fetal death (IUFD) and intra-uterine growth retardation (IUGR). However, the exact mechanism for LPS-induced IUFD and IURD remains unclear. LPS stimulates macrophages to generate reactive oxygen species (ROS). Therefore, we hypothesize that ROS may be involved in LPS-induced IUFD and IURD. Melatonin is a powerful endogenous antioxidant. In this study, we investigated the protective effects of melatonin on LPS-induced IUFD and IURD in ICR mice. All pregnant mice except controls received an intraperitoneal (75 μg/kg, i.p.) injection of LPS on gestational day (gd) 15–17. The experiment was carried out in two different modes. In mode A, the pregnant mice received two doses of melatonin within 24 hr, one (5 or 10 mg/kg) injected immediately after LPS and the other (5 or 10 mg/kg) injected at 3 hr after LPS. In mode B, the pregnant mice were pretreated with 10 mg/kg of melatonin 18 hr before LPS and then received two doses of melatonin in 24 hr, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. The number of live fetuses, dead fetuses and resorption sites were counted on gd 18. Live fetuses in each litter were weighed. Crown-rump and tail lengths were examined and skeletal development was evaluated. Results showed that post-treatments with melatonin significantly attenuated LPS-induced IUFD in a dose-dependent manner. Surprisingly, pre- plus post-treatments with melatonin almost blocked LPS-induced IUFD. In addition, both post-treatments and pre- plus post-treatments with melatonin significantly alleviated LPS-induced decreases in crown-rump and tail lengths and reversed LPS-induced skeletal developmental retardation. However, melatonin had little effect on LPS-induced decrease in fetal weight. Furthermore, pre- plus post-treatments with melatonin significantly attenuated LPS-induced lipid peroxidation in maternal liver. These results indicate that melatonin protects against LPS-induced IURD and IUGR via counteracting LPS-induced oxidative stress.

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Key words: intra-uterine fetal death, intra-uterine growth retardation, lipopolysaccharide, melatonin, oxidative stress

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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 [1]. Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal distress and alcohol consumption often increase the uptake of LPS from gastrointestinal tract into blood [2]. LPS has been associated with adverse developmental outcome, including intra-uterine fetal death (IUFD) and intra-uterine growth retardation (IURD) in rodents. In human, Gram-negative bacterial infections are a recognized cause of fetal loss and preterm labor [3–5]. Tumor necrosis factor alpha (TNF-α) appears to be an important mediator of IUFD caused by LPS [6–8]. Cyclooxygenase 2-mediated eicosanoid production is also involved in LPS-induced IUFD [9]. In addition, inhibition of nitric oxide (NO) production rescued LPS-induced fetal abortion [10]. Aminoguanidine, an inhibitor of inducible nitric oxide synthase (iNOS) activity, totally reversed the LPS-induced embryonic resorption [11]. A recent study showed that N-acetylcysteine, a glutathione (GSH) precursor and direct antioxidant, protected against fetal death and preterm labor induced by maternal inflammation [12], suggesting that reactive oxygen species (ROS) may mediate LPS-induced IUFD and IUGR.

Melatonin (N-acetyl-5-methoxytryptamine, melatonin), the major product of the pineal gland, plays a fundamental role in the neuroimmuno-endocrine system. As a potent antioxidant, melatonin directly scavenges hydroxyl free radicals ($^{\bullet}OH$) and peroxynitrite anion ($ONOO^{-}$) [13–16]. Melatonin also decreases free radical levels by stimulating the activities of enzymes involved in antioxidative defense [17–19]. In vivo, melatonin prevents circulatory failure in rats with endotoxemia and improves survival in mice treated with a lethal dose of LPS via inhibiting the release

of plasma TNF-α and the expression of hepatic iNOS [20]. Furthermore, melatonin protects mice against LPS-induced liver injury [21]. Recently, we found that melatonin attenuates LPS-induced downregulation of hepatic pregnane X receptor and CYP3A expression via counteracting LPS-induced oxidative stress [22].

In this study, we investigated the protective effects of melatonin on LPS induced IUFD and IUGR in ICR mice. Our results found that maternal melatonin administration protects against LPS-induced IUFD and reversed LPS-induced growth and skeletal development retardation via counteracting LPS-induced oxidative stress.

Materials and methods

Chemicals

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and melatonin were purchased from Sigma Chemical Co. (St Louis, MO, USA). All the other reagents were from Sigma or as indicated in the specified methods.

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-hr 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 21:00 hr. Females were checked by 7:00 hr the next morning, and the presence of a vaginal plug was designated as gestational day (gd) 0. The present study included two separate experiments.

Experiment 1: The pregnant mice were divided randomly into six groups. All pregnant mice except controls (either saline or melatonin) received an intraperitoneal (75 μg/kg, i.p.) injection of LPS between 08:00 and 09:00 hr on gd 15–17. The experiment was carried out in two different modes. In Mode A, the pregnant mice received two doses of melatonin in 24 hr, one (5 or 10 mg/kg) injected immediately after LPS and the other (5 or 10 mg/kg) injected at 3 hr after LPS. In Mode B, the pregnant mice were pretreated with 10 mg/kg of melatonin 18 hr before LPS and then received two doses of melatonin in 24 hr, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. All dams were killed 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. Crown-rump and tail lengths were measured. All fetuses were then stored in ethanol a minimum of 2 wk for subsequent skeletal evaluation.

Experiment 2: The pregnant mice were divided randomly into five groups. All pregnant mice except controls (either saline or melatonin) received an intraperitoneal (75 μg/kg, i.p.) injection of LPS between 08:00 and 09:00 hr on gd 15. In melatonin-treated group, the pregnant mice were divided into two subgroups depending on the schedule of melatonin administration: in Group M1, the pregnant mice received two doses of melatonin, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS; in Group M2, the pregnant mice were pretreated with 10 mg/kg of melatonin 18 hr before LPS and then received two doses of melatonin, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. All dams were killed at 6 hr after LPS treatment. Maternal liver and placenta were dissected for GSH and TBARS measurements. Maternal serum and amniotic fluid were collected for nitrite plus nitrate analyses.

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.

Skeletal examination and evaluation

The fetuses stored in ethanol were cleared of 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 ossification of the phalanges, metacarpals, vertebrae, sternatrae and skull. The size of the anterior fontanel and ossification of the supraoccipital was scored.

Determination of glutathione content

The GSH was determined by the method of Griffith [23]. 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 4500 g 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 protein. Protein content was measured according to the method of Lowry et al. [24].

Lipid peroxidation assay

Lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (TBARS) as described previously [25]. Maternal liver and placenta were homogenized in nine 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

Table 1. The effects of lipopolysaccharide (LPS) and melatonin on fetal outcomes

Treatments	Litters	Gestational sacs	Live fetuses	Fetal resorptions (%)
Control	21	243	231	11 (4.5)
Melatonin (10 mg/kg)	10	128	121	6 (5.0)
LPS (75 μg/kg)	12	161	57	6 (5.0)
LPS (75 μg/kg) + melatonin (5 + 5 mg/kg)	7	90	52	2 (2.2)
LPS (75 μg/kg) + melatonin (10 + 10 mg/kg)	11	148	92	7 (4.7)
LPS(75 μg/kg) + melatonin (10 + 10 + 10 mg/kg)	9	138	119	12 (8.7)

at 95°C for 1 hr. 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.

Analysis of nitrite plus nitrate concentration

The stable end products of L-arginine-dependent NO synthesis, nitrate and nitrite, were measured in maternal serum and amniotic fluid using a colorimetric method based on the Griess reaction [26, 27]. Briefly, aliquots of serum or amniotic fluid were added to 35% sulfosalicylic acid and vortexed every 5 min for 30 min to deproteinize samples. The samples were then centrifuged at 10,000 × g at 4°C for 15 min. An aliquot of the supernatant was taken for nitrite and nitrate analysis. Twenty microliters of plasma 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 hr 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°C. 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., Winooski, VT, USA) and a sodium nitrite standard curve.

Statistical analysis

Quantified data were expressed as mean ± S.E.M. at each point. Binomial data were analyzed using χ^2 analysis or Fisher's exact test where appropriate. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences between the treated animals and the control and level of statistical significance.

Results

Fetal outcomes were presented in Table 1. Results show that the incidence of resorptions did not differ among different groups. LPS and melatonin exhibited no obvious maternal side effects (data not shown). The protective effects of melatonin on LPS-induced IUFD were presented in Fig. 1. Maternal LPS administration on gd 15–17 resulted in 60.9% fetal death. Post-treatments with melatonin significantly attenuated LPS-induced IUFD in a dose-dependent manner. Pre- plus post-treatments almost totally blocked LPS-induced IUFD.

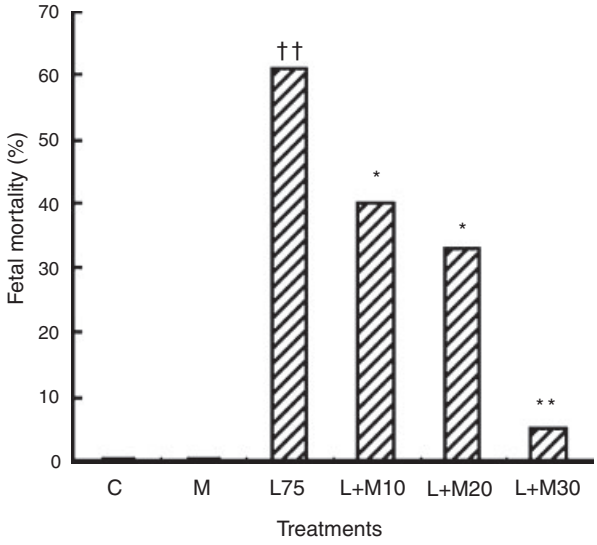

Fig. 1. The effects of melatonin on lipopolysaccharide (LPS)-induced intra-uterine fetal death. All pregnant mice except controls (either saline or melatonin) received an intraperitoneal (75 μg/kg, i.p.) injection of LPS on gestational day (gd) 15–17. In Mode A, the pregnant mice received two doses of melatonin in 24 hr, one (5 or 10 mg/kg) injected immediately after LPS and the other (5 or 10 mg/kg) injected at 3 hr after LPS. In Mode B, the pregnant mice were pretreated with 10 mg/kg of melatonin on gd 14 and then received two doses of melatonin in 24 h, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. The number of live fetuses and dead fetuses was counted on gd 18. C, control; M, melatonin; LPS, LPS (75 μg/kg/day); L + M10, LPS (75 μg/kg/day) plus melatonin (5 + 5 mg/kg in 24 hr); L + M20, LPS (75 μg/kg/day) plus melatonin (10 + 10 mg/kg in 24 hr); L + M30, pretreatment with melatonin (10 mg/kg) + LPS (75 μg/kg) + post-treatments with melatonin (10 + 10 mg/kg in 24 hr); ††$P < 0.01$ as compared with control group. *$P < 0.05$, **$P < 0.01$ as compared with LPS-treated group.

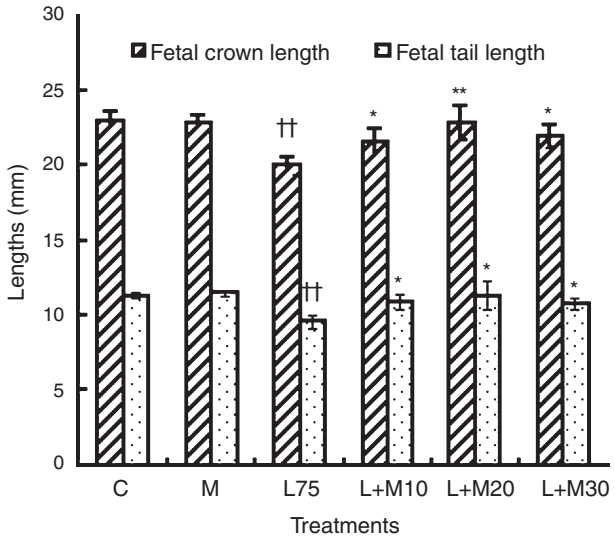

Fig. 2. The effects of melatonin on lipopolysaccharide (LPS)-induced decrease in fetal crown-rump and tail lengths. All pregnant mice except controls (either saline or melatonin) received an intraperitoneal (75 μg/kg, i.p.) injection of LPS on gestational day (gd) 15–17. In Mode A, the pregnant mice received two doses of melatonin in 24 hr, one (5 or 10 mg/kg) injected immediately after LPS and the other (5 or 10 mg/kg) injected at 3 hr after LPS. In Mode B, the pregnant mice were pretreated with 10 mg/kg of melatonin on gd 14 and then received two doses of melatonin in 24 hr, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. Fetal crown-rump and tail lengths were measured on gd 18. C, control; M, melatonin; LPS, LPS (75 μg/kg/day); L + M10, LPS (75 μg/kg/day) plus melatonin (5 + 5 mg/kg in 24 hr); L + M20, LPS (75 μg/kg/day) plus melatonin (10 + 10 mg/kg in 24 hr); L + M30, pretreatment with melatonin (10 mg/kg) + LPS (75 μg/kg) + post-treatments with melatonin (10 + 10 mg/kg in 24 hr); ††$P < 0.01$ as compared with control group. *$P < 0.05$, **$P < 0.01$ as compared with LPS-treated group.

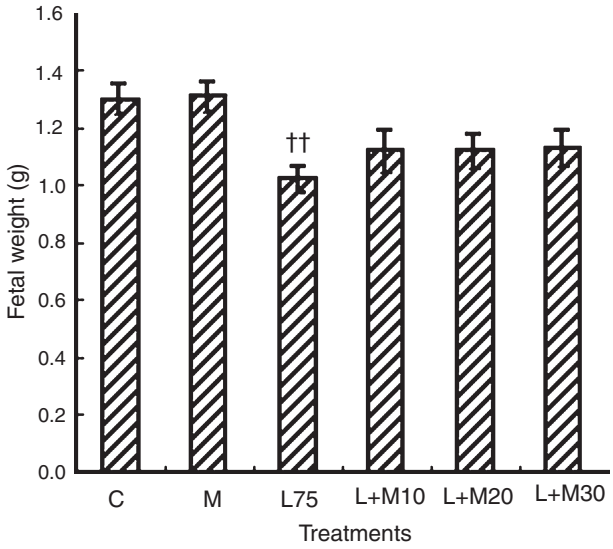

Fig. 3. The effects of melatonin on LPS-induced decrease in fetal weight. All pregnant mice except controls (either saline or melatonin) received an intraperitoneal (75 μg/kg, i.p.) injection of lipopolysaccharide (LPS) on gestational day (gd) 15–17. In Mode A, the pregnant mice received two doses of melatonin in 24 hr, one (5 or 10 mg/kg) injected immediately after LPS and the other (5 or 10 mg/kg) injected at 3 hr after LPS. In Mode B, the pregnant mice were pretreated with 10 mg/kg of melatonin on gd 14 and then received two doses of melatonin in 24 hr, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. Fetal weights were measured on gd 18. C, control; M, melatonin; LPS, LPS (75 μg/kg/day); L + M10, LPS (75 μg/kg/day) plus melatonin (5 + 5 mg/kg in 24 hr); L + M20, LPS (75 μg/kg/day) plus melatonin (10 + 10 mg/kg in 24 hr); L + M30, pretreatment with melatonin (10 mg/kg) + LPS (75 μg/kg) + post-treatments with melatonin (10 + 10 mg/kg in 24 h); ††$P < 0.01$ as compared with control group.

The protective effects of melatonin on LPS-induced reductions in crown-rump and tail lengths were presented in Fig. 2. Results showed that there were significant decreases in crown-rump and tail lengths in LPS-treated group as compared with the control. Both post-treatments and pre- plus post-treatments with melatonin significantly attenuated LPS-induced decreases in crown-rump and tail lengths. The effects of LPS on fetal weight were presented in Fig. 3. As expected, maternal LPS administration on gd 15–17 markedly decreased fetal weight. However, Both post-treatments and pre- plus post-treatments had little effect on LPS-induced decrease in fetal weight.

The effects of melatonin on LPS-induced skeletal developmental retardation were presented in Table 2. There were fewer ossification centers in caudal vertebrae, anterior and posterior phalanges in LPS-treated mice than those in the control. Post-treatments with melatonin significantly attenuated LPS-induced skeletal development retardation. Pre- plus post-treatments restored the number of ossification centers to control level. In addition, Pre- plus post-treatments with melatonin also reversed LPS-induced supraoccipital ossification retardation.

Lipid peroxidation was quantified by measuring TBARS. As shown in Fig. 4, LPS markedly increased TBARS levels in maternal liver and placenta. Pre- plus post-treatments with melatonin significantly attenuated LPS-induced lipid peroxidation in maternal liver. However, Pre- plus post-treatments with melatonin had little effect on LPS-induced lipid peroxidation in placenta. Furthermore, post-treatments did not alleviate LPS-induced lipid peroxidation in maternal liver and placenta.

The effects of melatonin on LPS-induced GSH depletion in maternal liver are presented in Fig. 5. Results showed that LPS significantly decreased GSH content in maternal liver. However, melatonin had little effect on LPS-induced GSH depletion.

Discussion

Lipopolysaccharide has been associated with adverse developmental outcome, including IUFD and IUGR, in rodents. The present study showed that maternal LPS treatment on gd 15–17 resulted in 60.9% fetal death. In addition, LPS also significantly reduced fetal weight, reduced crown-rump and tail lengths, and retarded skeletal ossification in caudal vertebrae, anterior and posterior phalanges, and supraoccipital bone. These results are in

Table 2. The effects of melatonin on lipopolysaccharide (LPS)-induced skeletal development retardation

	C (n = 21)	M (n = 10)	L75 (n = 12)	L + M10 (n = 7)	L + M20 (n = 11)	L + M30 (n = 9)
No. examined						
Fetuses	231	121	57	52	92	119
Scores						
Supraoccipital bone[a]	1.02 ± 0.01	1.10 ± 0.03	2.02 ± 0.25††	1.33 ± 0.20**	1.35 ± 0.21**	1.21 ± 0.10**
Number ossified:						
Sternum	6.00 ± 0.00	5.94 ± 0.25	5.87 ± 0.26	5.97 ± 0.04	5.99 ± 0.04	5.97 ± 0.05
Metacarpus	4.00 ± 0.00	3.95 ± 0.02	3.88 ± 0.29†	4.00 ± 0.00*	3.94 ± 0.17	4.00 ± 0.00*
Anterior phalanx	4.00 ± 0.00	3.95 ± 0.10	3.08 ± 1.42†	3.93 ± 0.16**	3.76 ± 0.59*	3.68 ± 0.48*
Metatarsus	4.98 ± 0.01	4.60 ± 0.30	4.56 ± 0.39†	4.71 ± 0.14	4.81 ± 0.23	4.88 ± 0.13*
Posterior phalanx	4.95 ± 0.02	3.80 ± 0.74	2.99 ± 2.09††	3.91 ± 0.53	3.91 ± 1.16	3.71 ± 0.86
Caudal vertebrae	6.30 ± 0.21	5.50 ± 0.85	3.67 ± 0.91††	4.37 ± 0.76	4.32 ± 0.56**	4.21 ± 0.25

Numbers of litters in each group were presented in parentheses. C, control; M, melatonin (10 mg/kg); L75, LPS (75 μg/kg); L + M10, LPS (75 μg/kg) + melatonin (5 + 5 mg/kg); L + M20, LPS (75 μg/kg) + melatonin (10 + 10 mg/kg); L + M30, LPS (75 μg/kg) + melatonin (10 + 10 + 10 mg/kg).
[a]Supraoccipital bone scores: 1 = well ossified, 4 = completely unossified.
Significantly different from control †$P < 0.05$, ††$P < 0.01$.
Significantly different from LPS75 group *$P < 0.05$, **$P < 0.01$.

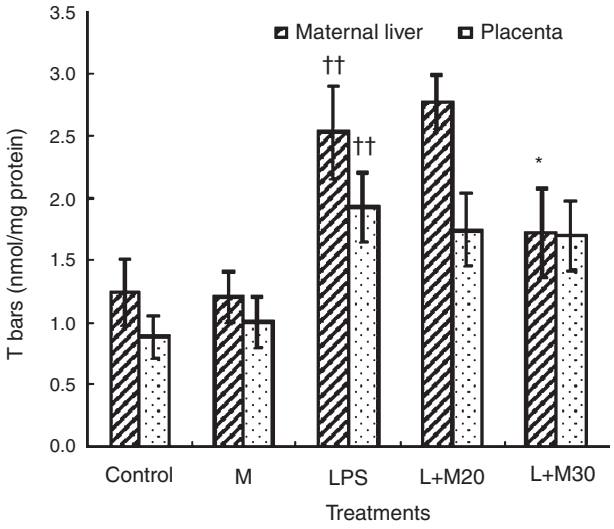

Fig. 4. The effects of melatonin on lipopolysaccharide (LPS)-induced lipid peroxidation. All pregnant mice except controls (either saline or melatonin) received an intraperitoneal (75 μg/kg, i.p.) injection of LPS on gestational day (gd) 15. In mode A, the pregnant mice received two doses of melatonin, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected at 3 hr after LPS. In mode B, the pregnant mice were pretreated with 10 mg/kg of melatonin on gd 14 and then received two doses of melatonin, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. TBARS level in maternal liver and placenta was analyzed as described in Materials and methods. Data were expressed as mean ± S.E.M. of eight mice in each point. C, control; M, melatonin; LPS, LPS (75 μg/kg); L + M20, LPS (75 μg/kg) plus melatonin (10 + 10 mg/kg); L + M30, pretreatment with melatonin (10 mg/kg) + LPS (75 μg/kg) + post-treatments with melatonin (10 + 10 mg/kg); ††$P < 0.01$ as compared with control group. *$P < 0.05$ versus LPS-treated control.

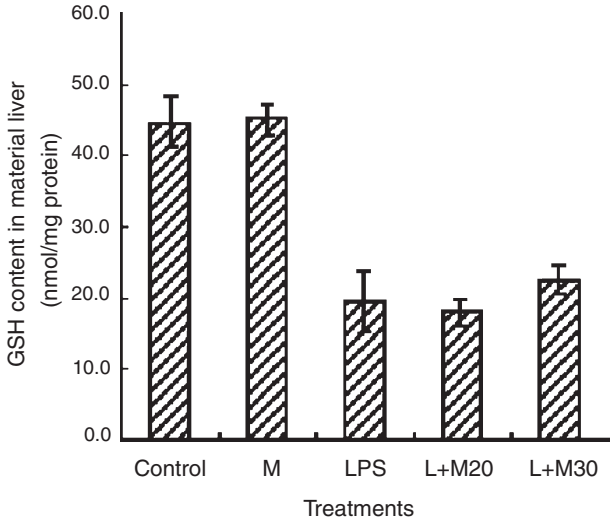

Fig. 5. The effects of melatonin on lipopolysaccharide (LPS)-induced glutathione (GSH) depletion. All pregnant mice except controls (either saline or melatonin) received an intraperitoneal (75 μg/kg, i.p.) injection of LPS on gestational day (gd) 15. In mode A, the pregnant mice received two doses of melatonin, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected at 3 hr after LPS. In mode B, the pregnant mice were pretreated with 10 mg/kg of melatonin on gd 14 and then received two doses of melatonin, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. GSH content in maternal liver was analyzed as described in Materials and methods. Data were expressed as mean ± S.E.M. (n = 8). C, control; M, melatonin; LPS, LPS (75 μg/kg); L + M20, LPS (75 μg/kg) plus melatonin (10 + 10 mg/kg); L + M30, pretreatment with melatonin (10 mg/kg) + LPS (75 μg/kg) + post-treatments with melatonin (10 + 10 mg/kg); ††$P < 0.01$ as compared with control group.

agreement with earlier work of Revera et al. [5], in which administration with 100 μg/kg LPS to rats on gd 14–20 caused 43% fetal death and reduced the size of the surviving fetuses.

To this date, the exact mechanism for LPS-induced IUFD and IURD remained unclear. Several studies indicated that LPS stimulates macrophages to generate ROS and increases nitrotyrosine, a marker for $O_2^{\bullet-}$, NO and

$ONOO^-$ formation, in macrophage-rich organs [28, 29]. Ejima et al. [30, 31] reported that LPS enhanced placental expression of 4-hydroxy-2-nonenal (HNE)-modified proteins, markers of oxidative stress. Furthermore, our earlier study found that alpha-phenyl-N-t-butylnitrone (PBN), a free radical spin-trapping agent, blocked LPS-induced IUFD and reversed LPS-induced growth and skeletal development retardation (data not shown). Therefore, we hypothesize that ROS may be involved in LPS-induced IUFD and IURD.

Melatonin is a powerful scavenger of oxygen free radicals [16]. In humans, melatonin is rapidly transferred from the maternal to the fetal circulation [32]. In the present study, we investigated the protective effect of melatonin on LPS-induced IUFD and IUGR. In Mode A, the pregnant mice received two doses of melatonin in 24 hr, one (5 or 10 mg/kg) injected immediately after LPS and the other (5 or 10 mg/kg) injected at 3 hr after LPS. In Mode B, the pregnant mice were pretreated with 10 mg/kg (i.p.) of melatonin 18 hr before LPS and then received two doses of melatonin, one (10 mg/kg) injected immediately after LPS and the other (10 mg/kg) injected 3 hr after LPS. Results indicate that melatonin post-treatments significantly attenuated LPS-induced IUFD in a dose-dependent manner. Surprisingly, pre- plus post-treatments almost completely blocked LPS-induced IUFD. Furthermore, both post-treatments and pre- plus post-treatments with melatonin markedly alleviated LPS-induced decreases in crown-rump and tail lengths and reversed LPS-induced skeletal development retardation. However, melatonin had little effect on LPS-induced decrease in fetal weight.

The antioxidative effect of melatonin can occur by at least three mechanisms [33, 34]. First, melatonin itself exerts direct antioxidative effects via scavenging free radicals and inhibiting free radical generation [35]. Additionally, melatonin alters the activities of antioxidative enzymes, which improve the endogenous antioxidative defense capacity of organisms [19]. Finally, melatonin decreased the iNOS gene expression and inhibited LPS-induced NO release [36]. Furthermore, metabolites that are formed when melatonin functions as a scavenger, i.e. N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) [37] and N^1-acetyl-5-methoxykynuramine (AMK) [38], are also highly effective scavengers which contribute to melatonin's antioxidative functions [39, 40].

The ability of melatonin to scavenge free radicals is undoubtedly an important property in its protection against oxidative stress. Excessive formation and release of ROS such as NO, superoxide, hydroxyl radical and hydrogen peroxide represent the predominant components of LPS-induced inflammatory events [28–31]. Several studies showed that melatonin directly scavenges hydroxyl free radicals ($^{\bullet}OH$) to form cyclic 3-hydroxymelatonin (3-OHM) [41, 42]. In addition, melatonin has also been shown to scavenge peroxynitrite anion ($ONOO^-$), the highly destructive product of the interaction between the superoxide anion radical ($O_2^{\bullet -}$) and NO [43, 44]. In the present study, we investigated the effects of melatonin on LPS-induced lipid peroxidation in maternal liver and placenta. Results indicated that melatonin significantly attenuated LPS-induced increases in TBARS in maternal liver. These results suggest that melatonin alleviated LPS-induced IUFD and IUGR via counteracting LPS-induced lipid peroxidation.

The effects of melatonin on the activities of antioxidative enzymes have been extensively studied [19]. According to a recent report, a single dose of melatonin (5 mg/kg) enhanced SOD and GSH-Rd activities in rat liver [45]. Melatonin also attenuated the decrease in hepatic Cu,Zn-SOD and GSH-Rd activities without affecting hepatic CAT and GSH-Px activities in alpha-naphthylisothiocyanate-treated rats [46]. A recent study found that melatonin attenuated CCl_4-induced reductions in hepatic SOD, CAT and GSH-Rd activities in a dose-dependent manner without affecting hepatic GSH-Px activity in CCl_4-treated rats [47]. SOD and CAT are two important antioxidative enzymes. SOD dismutases $O_2^{-\bullet}$ to H_2O_2, decreasing the amount of $O_2^{\bullet -}$ and the formation of $ONOO^-$. CAT then eliminates H_2O_2. In our earlier study, we investigated the effects of melatonin on hepatic SOD and CAT activities in LPS-treated mice. Results indicated that melatonin significantly increased hepatic SOD and CAT activities in LPS-treated mice [22]. GSH-Px and GSH-Rd are involved in GSH metabolism. GSH-Px transforms H_2O_2 to O_2. In this process GSH is oxidized, thus forming oxidized GSSG. The reduced GSH is replenished by the action of GSH-Rd. Our earlier results showed that melatonin significantly increased hepatic GSH-Px and GSH-Rd activities in LPS-treated mice [22]. In the present study, we measured GSH content in maternal liver, placenta and fetal liver. Results showed that LPS significantly decreased GSH content in maternal liver. However, both post-treatments and pre- plus post-treatments with melatonin did not influence hepatic reduced GSH content in LPS-treated pregnant mice. These results suggest that the protective effect of melatonin on LPS-induced IUFD and IUGR did not attribute to GSH metabolism.

Excess NO production plays a pivotal role on LPS-induced resorption and fetal abortion [10, 11]. Recent studies showed that melatonin decreased the iNOS mRNA level and inhibited iNOS activity in LPS-treated rats [35]. In the present study, we measured nitrite plus nitrate level in maternal serum and amniotic fluid at 6 hr after LPS treatment. Results showed that a single dose (75 μg/kg) of LPS did not increase nitrite plus nitrate concentration in maternal serum and amniotic fluid. These results suggest that protective effect of melatonin on LPS-induced IUFD and IUGR did not attribute to repression of NO production. TNF-α has been associated with LPS-induced developmental toxicity [5, 7, 8]. A recent study found that melatonin inhibited the release of TNF-α in plasma [48]. Thus, our results do not exclude that melatonin attenuated LPS-induced IUFD and IUGR by repression of TNF-α production.

In summary, the present study found that maternal LPS exposure significantly increased fetal mortality, decreased fetal weights and crown-rump lengths, and retarded skeletal ossification in caudal vertebrae, anterior and posterior phalanges, and supraoccipital bone. Melatonin significantly attenuated LPS-induced IUFD and reversed LPS-induced growth and skeletal development retardation via counteracting LPS-induced oxidative stress.

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