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Maternally administered melatonin differentially regulates lipopolysaccharide-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain

Abstract: Lipopolysaccharide (LPS) has been associated with adverse developmental outcome, including intra-uterine fetal death and intra-uterine growth retardation. In the LPS model, tumor necrosis factor alpha (TNF-α) is the major mediator leading to intra-uterine fetal death and intra-uterine growth retardation. Interleukin (IL)-10 protects rodents against LPS-induced intra-uterine fetal death and intra-uterine growth retardation. Melatonin is an immunomodulator. In the present study, we investigated the effect of maternally administered melatonin on LPS-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver and fetal brain. The time pregnant mice were injected with melatonin [5.0 mg/kg, intraperitoneal (i.p.)] 30 min before LPS (500 μg/kg, i.p.) on gestational day 17. As expected, TNF-α, IL-1β, IL-6 and IL-10 were obviously increased in maternal serum and amniotic fluid in response to LPS. In addition, maternal LPS exposure significantly increased the levels of TNF-α, IL-1β, IL-6 and IL-10 in fetal liver, and TNF-α and IL-10 in fetal brain. Melatonin pretreatment significantly attenuated LPS-evoked elevation of TNF-α in maternal serum. On the contrary, melatonin aggravated LPS-induced increase in IL-10 in maternal serum. Melatonin had no effect on LPS-evoked IL-1β and IL-6 in maternal serum and amniotic fluid. Interestingly, maternally administered melatonin also significantly attenuated LPS-evoked elevation of TNF-α in fetal brain, whereas the indole aggravated LPS-induced increase in IL-10 in fetal liver. Taken together, these results indicate that maternally administered melatonin differentially regulates LPS-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain.

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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 the gastrointestinal tract into blood [2]. LPS has been associated with adverse developmental outcome, including embryonic resorption, intra-uterine fetal death, intra-uterine growth restriction, and preterm labor and delivery in rodents [3–6]. In the LPS model, tumor necrosis factor alpha (TNF-α) is one of the major mediators leading to embryonic resorption, intra-uterine fetal death, intra-uterine growth retardation and preterm delivery [7–9]. In contrast with TNF-α, interleukin (IL)-10 protects rodents against LPS-induced intra-uterine fetal death and intra-uterine growth retardation [10].

Melatonin (*N*-acetyl-5-methoxytryptamine), the major product of the pineal gland, plays a fundamental role in the neuroimmuno-endocrine system. As a potent antioxidant, melatonin and its metabolites directly scavenges hydroxyl free radicals ($^{\bullet}OH$) [11, 12] and peroxynitrite anion ($ONOO^{-}$) [13]. Numerous studies indicate that melatonin also decreases free radical levels by stimulating the activities of enzymes involved in antioxidative defense [14, 15]. An earlier study has demonstrated that melatonin was transferred from the maternal to the fetal circulation easily and rapidly [16]. Maternally administered melatonin protects against ischemia/reperfusion-induced oxidative damage to mitochondria in fetal rat brain [17, 18]. Recently, we have found that administration of melatonin to the pregnant mice protected against LPS-induced intra-uterine fetal death and intra-uterine growth retardation [19].

On the other hand, melatonin is also an immunomodulator [20–22]. In vivo, melatonin prevents circulatory failure in rats and in humans with endotoxemia and improve the survival of mice with septic shock by repression of proinflammatory cytokines [23–25]. Therefore, we hypothesize that the protective effect of melatonin on

LPS-induced intra-uterine fetal death and intra-uterine growth retardation is associated with its repression of proinflammatory cytokines. However, it is not clear whether maternal melatonin administration blocks LPS-induced release of proinflammatory cytokines in amniotic fluid, placenta, fetal liver, and fetal brain.

In the present study, we investigated the effect of maternally administered melatonin on LPS-evoked TNF-α, IL-1β, IL-6 and IL-10 in maternal serum, amniotic fluid, fetal liver, and fetal brain. We found that melatonin differentially regulates LPS-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain.

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- to 10-wk 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 wk 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 pregnant mice were divided randomly into four groups. All pregnant mice except controls (saline and melatonin) received an intraperitoneal (i.p.) injection of LPS (500 μg/kg) between 08:00 and 09:00 hr on gd 17. In melatonin + LPS group, the pregnant mice were pretreated with melatonin (5.0 mg/kg, i.p.) at 30 min before LPS administration. The control mice received saline or melatonin (5.0 mg/kg, i.p.). All pregnant mice were killed at 1.5 hr after LPS administration. Maternal serum and amniotic fluid were collected for measurement of cytokines. The fetal brain and fetal liver tissue (100 mg) was placed in 1 mL of iced lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, and 0.1 mM PMSF (recipe from Upstate, Charlottesville, VA, USA). Samples were homogenized and centrifuged at 10,500 g for 20 min at 4°C. Supernatants were aliquoted for measurement of cytokines. Protein concentrations of supernatant samples were measured according to the method of Lowry et al., using bovine serum albumin as a standard.

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.

Measurement for cytokine concentration

Commercial ELISA (R&D Systems, Abingdon, Oxon, UK) kits were used to determine levels of TNF-α, IL-1β, IL-6, and IL-10 in maternal serum, amniotic fluid, fetal liver and fetal brain according to the manufacturer's protocol. Briefly, samples were pipetted in wells precoated with specific antibody for mouse TNF-α, IL-1β, IL-6, or IL-10 and allowed to incubate for 2 hr. After wells were rinsed to remove all unbound substance, an enzyme-linked antibody specific for mouse TNF-α, IL-1β, IL-6, or IL-10 was added to wells for 2 hr. 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., Winooski, VT, USA). For all kits used, the minimum detectable limit was < 10 pg/mL. The mean intra-assay and inter-assay variations for TNF-α, IL-1β, IL-6, and IL-10 were $< 10\%$.

Statistical analysis

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. Differences were considered to be significant only for $P < 0.05$.

Results

First, we investigated the effects of melatonin on LPS-evoked cytokines in maternal serum. In response to LPS, TNF-α, IL-1β, IL-6 and IL-10 in maternal serum were significantly increased 1.5 hr after LPS administration. Melatonin pretreatment significantly attenuated LPS-evoked elevation of TNF-α in maternal serum. On the contrary, melatonin pretreatment aggravated the elevation of IL-10 in maternal serum of LPS-treated pregnant mice. Melatonin had no effect on LPS-evoked elevation of IL-1β and IL-6 in maternal serum (Fig. 1).

The effects of melatonin on LPS-evoked cytokines in amniotic fluid are presented in Fig. 2. Results showed that TNF-α and IL-6 in amniotic fluid were obviously increased 1.5 hr after maternal LPS administration. Melatonin pretreatment significantly attenuated LPS-evoked elevation of TNF-α in amniotic fluid. However, melatonin had no effect on LPS-evoked increase in IL-6 in amniotic fluid. IL-10 in amniotic fluid showed a significant elevation in response to LPS. However, melatonin pretreatment had no effect on LPS-induced increase in IL-10 in amniotic fluid.

The effects of melatonin on LPS-evoked cytokines in fetal liver were shown in Fig. 3. Results showed that maternal LPS exposure significantly increased the levels of TNF-α, IL-1β, IL-6 and IL-10 in fetal liver. Melatonin pretreatment aggravated LPS-induced elevation of IL-10 in fetal liver. However, maternally administered melatonin had no effect on LPS-evoked elevation of TNF-α, IL-1β and IL-6 in fetal liver.

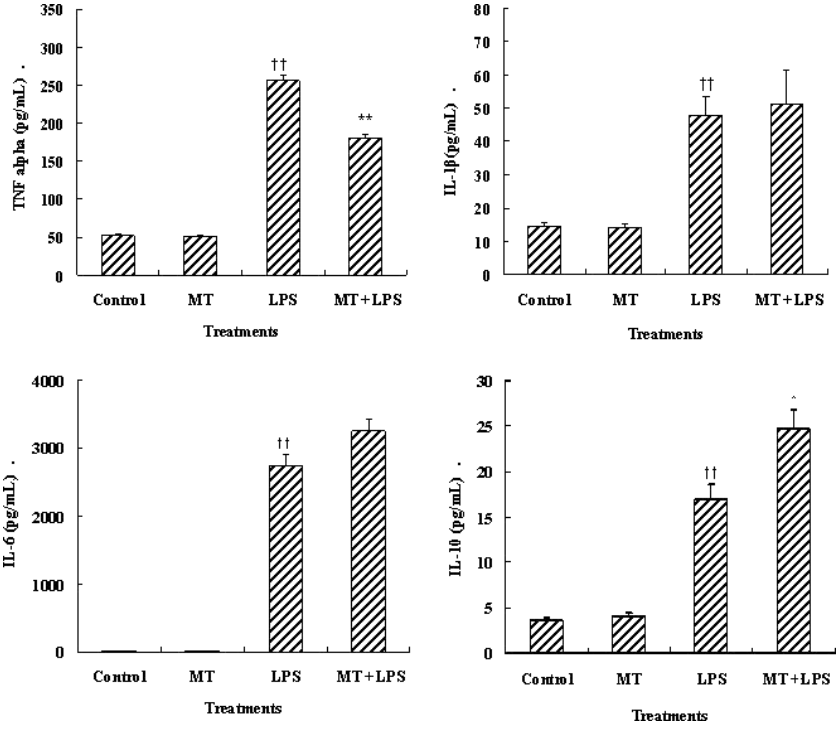


Fig. 1. The effects of melatonin on lipopolysaccharide (LPS)-evoked cytokine induction in maternal serum. The pregnant mice were pretreated with melatonin (5.0 mg/kg) at 30 min before LPS (500 μg/kg). Tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-10 in maternal serum was measured at 1.5 hr after LPS. Data were expressed as mean ± S.E.M. (n = 12). †† $P < 0.01$ as compared with control group. *$P < 0.05$, **$P < 0.01$ as compared with LPS group.

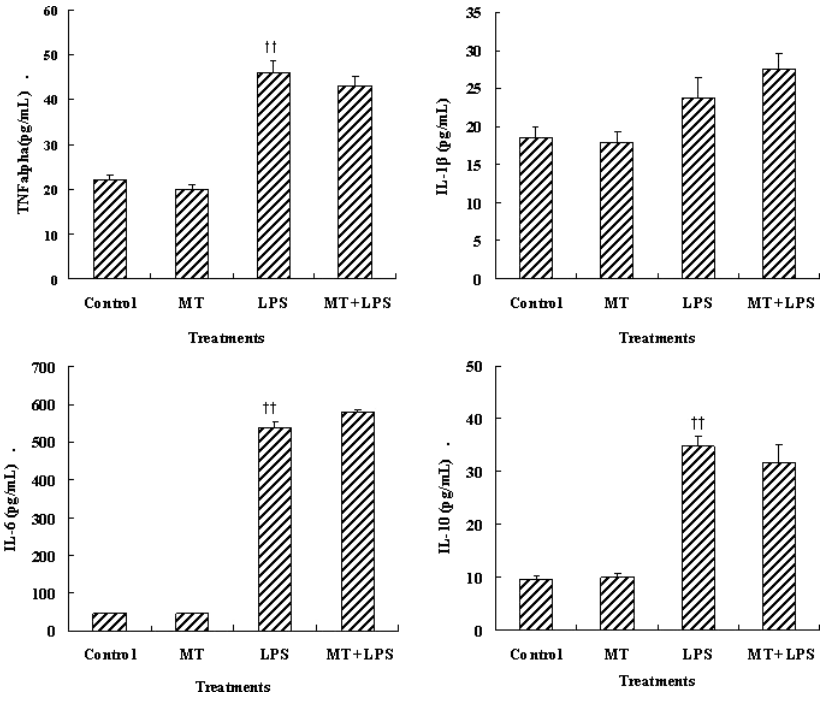
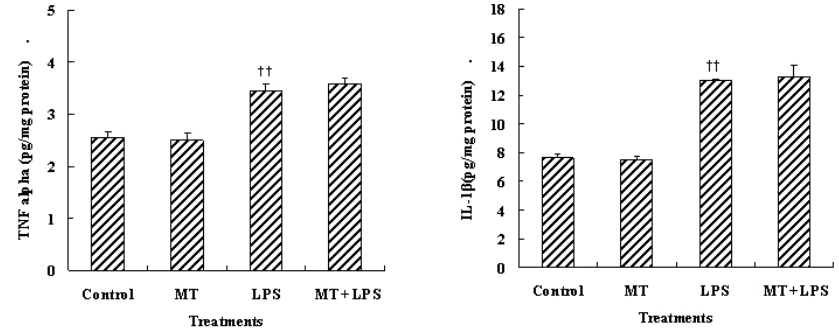


Fig. 2. The effects of melatonin on lipopolysaccharide (LPS)-evoked cytokine induction in amniotic fluid. The pregnant mice were pretreated with melatonin (5.0 mg/kg) at 30 min before LPS (500 μg/kg). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 in amniotic fluid was measured at 1.5 hr after LPS. Data were expressed as mean ± S.E.M. (n = 12). †† $P < 0.01$ as compared with control group.

Finally, we analyzed the effects of melatonin on LPS-evoked cytokines in fetal brain. As shown in Fig. 4, maternal LPS exposure significantly increased TNF-α and IL-10 in fetal brain. By contrast, maternal LPS exposure significantly decreased IL-1β in fetal brain. Melatonin significantly reduced the levels of TNF-α, IL-6 and IL-10 in fetal brain. Melatonin had no effect on IL-1β in fetal brain.

Discussion

Lipopolysaccharide has been associated with adverse developmental outcome, including embryonic resorption, intra-uterine fetal death, intra-uterine growth restriction, and preterm labor and delivery in rodents [6, 7, 9, 10, 15, 26, 27]. Recently, we found that maternally administered melatonin effectually protects mice against LPS-induced



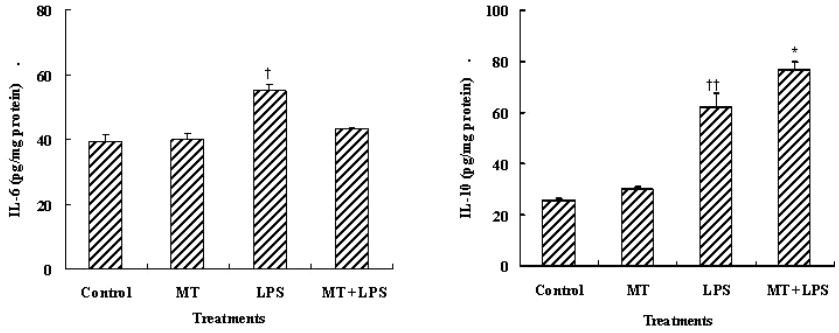
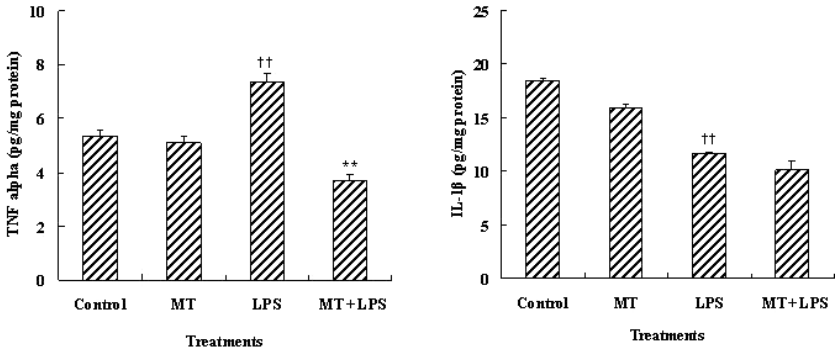


Fig. 3. The effects of melatonin on lipopolysaccharide (LPS)-evoked cytokine induction in fetal liver. The pregnant mice were pretreated with melatonin (5.0 mg/kg) at 30 min before LPS (500 μg/kg). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 in fetal liver was measured at 1.5 hr after LPS. Data were expressed as mean ± S.E.M. (n = 12). † $P < 0.05$, †† $P < 0.01$ as compared with control group. *$P < 0.05$ as compared with LPS group.



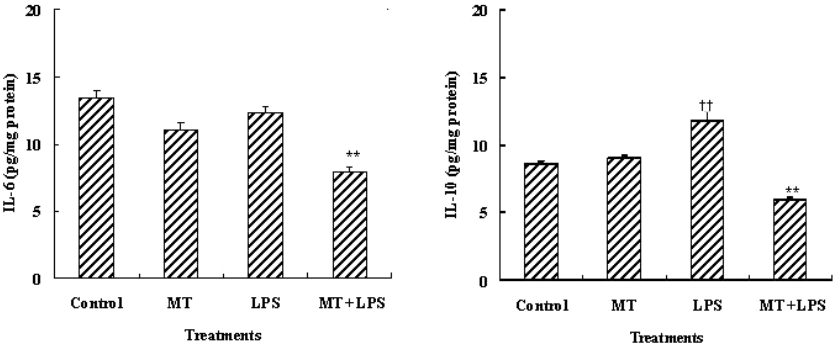


Fig. 4. The effects of melatonin on lipopolysaccharide (LPS)-evoked cytokine induction in fetal brain. The pregnant mice were pretreated with melatonin (5.0 mg/kg) at 30 min before LPS (500 μg/kg). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 in fetal brain was measured at 1.5 hr after LPS. Data were expressed as mean ± S.E.M. (n = 12). †† $P < 0.01$ as compared with control group. **$P < 0.01$ as compared with LPS group.

intra-uterine fetal death and intra-uterine growth restriction and reversed LPS-induced skeletal developmental retardation [19]. In the LPS model, TNF-α is one of the major mediators leading to embryonic resorption, intra-uterine fetal death, intra-uterine growth retardation and preterm delivery [7–9]. The present study showed that administration of a single dose of LPS obviously increased release of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in maternal serum and amniotic fluid. These results are in agreement with earlier work of Gayle et al. [28], in which TNF-α, IL-1β and IL-6 were elevated in maternal serum and amniotic fluid of LPS-treated pregnant rats. Several studies showed that melatonin, as an immunomodulator, inhibited LPS-evoked release of proinflammatory cytokines [20–23, 25]. Therefore, it was especially interesting to determine whether the protective effect of melatonin on LPS-induced developmental toxicity is associated with its anti-inflammatory actions. In the present study, we

investigated the effects of melatonin on TNF-α in LPS-treated pregnant mice. Interestingly, maternally administered melatonin significantly attenuated LPS-evoked elevation of TNF-α in maternal serum. These results suggest that the protective effects of melatonin on LPS-induced developmental toxicity might be, at least in part, due to its repression of TNF-α in maternal serum and amniotic fluid. The present study also investigated the effects of melatonin on IL-6 and IL-1β in LPS-treated pregnant mice. We found that melatonin had no effect on releases of IL-1β and IL-6 in maternal serum and amniotic fluid. The cause may be probably due to the short time analyzed after its administration, because the animals were killed 1.5 hr after LPS administration. In addition, the dose of melatonin used for anti-septic effects of melatonin was 10 mg/kg [19, 25, 29]. Therefore, the low dose of melatonin used, i.e. 5 mg/kg, may be another explanation for its lack of effect.

Interleukin-10 is an important anti-inflammatory cytokine. In the present study, we investigated the effect of melatonin on the releases of IL-10 in maternal serum and amniotic fluid of LPS-treated pregnant mice. As expected, there was a significant rise in IL-10 in maternal serum and amniotic fluid in response to LPS challenge. Interestingly, melatonin pretreatment aggravated LPS-evoked elevation of IL-10 in maternal serum, whereas maternally-administered melatonin had no effect on the release of IL-10 in amniotic fluid of LPS-treated pregnant mice. Several studies showed that IL-10 protected against LPS-induced intra-uterine fetal death, intra-uterine growth restriction and preterm labor [10, 30]. Taken together, these results suggest that the protective effects of melatonin on LPS-induced developmental toxicity might also be associated with an elevation of IL-10 in maternal serum.

Kupffer cells are present in fetal liver. A recent study found that murine fetal liver expresses high levels of TLR-4 mRNA [31]. In vitro studies have demonstrated that fetal Kupffer cells secret TNF-α and IL-1β in response to LPS [32]. Our earlier studies indicated that maternal LPS exposure results in lipid peroxidation and GSH depletion in fetal liver [33]. In the present study, we investigated whether LPS induces the expression of proinflammatory and anti-inflammatory cytokines in fetal liver. Results showed that the levels of proinflammatory (TNF-α, IL-1β, and IL-6) and anti-inflammatory (IL-10) cytokines in fetal liver were significantly increased 1.5 hr after maternal LPS exposure. To investigate the effect of melatonin on LPS-induced proinflammatory and anti-inflammatory cytokines in fetal liver, the pregnant mice were pretreated with melatonin 30 min before LPS. Surprisingly, melatonin pretreatment aggravated LPS-induced elevation of anti-inflammatory cytokine IL-10 in fetal liver, whereas maternally administered melatonin had no effect on LPS-evoked induction of proinflammatory cytokines in fetal liver.

Several studies showed that maternal LPS exposure did not increase the expression of cytokines in fetal brain [28, 34]. However, Bell et al. [35] found that TNF-α was increased fivefold in fetal brain in response to maternally administered LPS. In the present study, we showed that there was a significant increase in TNF-α and IL-10 in fetal brain in response to maternal LPS challenge. Interestingly, maternally administered melatonin significantly reduced the levels of TNF-α in fetal brain.

In summary, the present study indicates that maternal infection increases the release of proinflammatory cytokines in maternal serum and amniotic fluid, which may contribute to LPS-induced intra-uterine fetal death, intra-uterine growth restriction and preterm labor. Importantly, maternal LPS exposure also stimulates TNF-α, IL-1β and IL-6 in fetal liver and fetal brain, suggesting that LPS-induced developmental toxicity may be not only a maternally mediated event. In addition, these cytokines may also contribute to aberrant brain development and offspring neurological disorders. Maternally administered melatonin differentially regulates LPS-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain. Melatonin pretreatment effectually inhibits LPS-evoked proinflammatory cytokine TNF-α in maternal serum, amniotic fluid and fetal brain, whereas this indole stimulates the releases of anti-inflammatory cytokine IL-10 in maternal serum and fetal liver of LPS-treated mice. An earlier study showed that maternally administered melatonin is not toxic to the fetuses [36]. Thus, melatonin may be used as pharmacological agents to protect the fetuses against LPS-induced intra-uterine fetal death, intra-uterine growth restriction, preterm labor, and neurological injury.

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