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Oral cholecalciferol supplementation alleviates lipopolysaccharide-induced preterm delivery partially through regulating placental steroid hormones and prostaglandins in mice

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ABSTRACT

Several epidemiological reports demonstrated that vitamin D deficiency elevated risk of preterm delivery. We investigate the effects of oral cholecalciferol (VD3) supplementation on lipopolysaccharide (LPS)-induced preterm delivery. Pregnant mice were randomly assigned to either oral VD3 (25 μg/kg) or corn oil once daily from gestational day (GD)13 to GD15, and were intraperitoneally injected with either LPS (200 μg/kg) or normal saline on GD15. As expected, LPS was effective in inducing preterm delivery and fetal death. LPS-induced preterm delivery and fetal death were alleviated in VD3-pretreated mice. LPS-induced down-regulation of genes for placental progesterone biosynthetic enzymes was blocked in VD3-pretreated mice. LPS-induced reduction of serum progesterone was correspondingly attenuated by VD3. Although oral VD3 had no effect on estradiol production, it attenuated LPS-induced up-regulation of placental ERβ in mice. LPS-induced placental COX-2 up-regulation and serum PGF2α elevation were alleviated in VD3-pretreated mice. Additionally, LPS-evoked elevations of the placental *Tnfα*, *Il1β*, *Mcp1* and *Mip2* mRNAs were attenuated by VD3. VD3 promoted placental vitamin D receptor nuclear translocation and simultaneously alleviated LPS-induced nuclear translocation of NF-κB p65 and p50 subunits. These results provide evidence that oral VD3 supplementation alleviates LPS-induced preterm delivery and fetal demise partially through regulating placental steroid hormones and prostaglandins.

1. Introduction

Lipopolysaccharide (LPS) is the toxic component of Gram-negative bacteria. In general, LPS is present in gastrointestinal tract of humans and animals and thus humans are constantly exposed to a low level of LPS [1]. Gastrointestinal inflammation and excess alcohol drinking impairs intestinal mucosal integrity, which promotes LPS translocation from intestinal cavity into portal system [2]. In addition, bacterial infection elevates the level of LPS in peripheral circulation [3]. Increasing evidence has demonstrated that LPS is associated with adverse pregnant outcomes. Several reports showed that maternal LPS exposure in the early gestational stage caused embryonic abortion and fetal demise in rodent animals [4–6]. Our studies demonstrated that LPS exposure during organogenesis resulted in neural tube defects in mouse fetuses [7,8]. In addition, maternal LPS exposure in the late gestational stage induced preterm delivery and fetal growth restriction in mice [9–12].

Vitamin D, a secosteroid hormone, is known for its classical functions in calcium uptake and bone metabolism [13]. Recently, increasing evidence demonstrates that gestational vitamin D deficiency has been associated with adverse pregnant outcomes. According to several epidemiological reports, gestational vitamin D deficiency elevated the risks of small for gestational age and low birth weight infants [14–16]. Moreover, gestational vitamin D deficiency elevated the risks of preterm delivery [17–19]. Therefore, it is especially interesting to test whether maternal cholecalciferol (Vitamin D3, VD3) supplementation alleviates LPS-induced adverse pregnant outcomes.

The aim of the present study is to investigate whether oral supplementation with VD3 attenuates LPS-induced preterm delivery in mice. Our results showed that oral VD3 supplementation promoted VDR nuclear translocation in mouse placenta. We found that oral VD3

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supplementation alleviated LPS-induced preterm delivery and fetal demise. We provide evidence for the first time that VDR-mediated alleviation on LPS-induced preterm delivery and fetal demise is partially attributed to its regulation of placental steroid hormones and prostaglandins.

2. Methods

2.1. Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127: B8) and VD3 (Cholecalciferol, C9756) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). StAR, 3βHSD, CYP11A1, CYP17A1, CYP19, ERα, ERβ, p65, p50, α-tubulin and Lamin A/C antbodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). p-IκBα, IκBα and VDR antibody was from Abcam (Cambridge, MA). Chemiluminescence (ECL) detection kits were from Pierce Biotechnology (Rockford, IL, USA). TRIzol reagent was from Molecular Research Center (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). Prostaglandin F2α (PGF2α) kits were from R&D Systems (Minneapolis, MN, USA). All the other reagents were from Sigma or as indicated in the specified methods.

2.2. Animals and treatments

The ICR mice (8–10 weeks old; male mice: 30–34 g; female mice: 26–30 g) were purchased from Beijing Vital River. The animals were maintained in a controlled environment (temperature: 20–25 °C, humidity: 50 ± 5%) on a 12 h light/dark cycle and permitted access to food and water ad libitum. All mice were continually fed a standard AIN93G rodent diet with 1000 IU VD3/kg (Trophic Animal Feed High-Tech Co., Ltd., China) [20]. For mating purposes, females were housed overnight with males at a 2:1 ratio at 9:00 P.M. Females were checked by 8:30 AM the next morning. The presence of a vaginal plug was designated as gestational day (GD) 0.

Experiment 1. To investigate the effects of oral VD3 supplementation on LPS-induced preterm delivery, all pregnant mice were divided into Control, VD3, LPS, and LPS + VD3 groups. Pregnant mice were randomly assigned to either oral VD3 supplementation (25 μg/kg, dissolved in corn oil) or corn oil once daily from GD13 to GD15. The dose of VD3 used was chosen to be similar to the doses used in previous studies [21]. All pregnant mice were intraperitoneally (i.p.) injected with either normal saline or LPS (200 μg/kg, dissolved in normal saline) on GD15. The dose of LPS used was chosen on basis of our previous study [22]. All pregnant mice were observed closely for signs of preterm delivery (such as vaginal bleeding or posture). The latency period was defined as the time from LPS or saline solution injection to the delivery of the first pup until GD19 [22].

Experiment 2. To analyze the effect of oral VD3 supplementation on LPS-induced placental steroid hormones' and prostaglandin's signaling, all pregnant mice were divided into Control, VD3, LPS, and LPS + VD3 groups. Pregnant mice were randomly assigned to either oral VD3 supplementation (25 μg/kg) or corn oil once daily from GD13 to GD15. The dose of VD3 used was chosen to be similar to the doses used in previous studies [21]. All pregnant mice were i.p. injected with either normal saline or LPS (200 μg/kg) on GD15. The dose of LPS used was chosen on basis of our previous study [22]. Six pregnant mice in each group were euthanized 6 h after LPS injection on GD15. Trunk blood was collected and serum prepared for biochemical assays. Some placentas were collected and frozen immediately in liquid nitrogen for real-time RT-PCR and Western blotting and other placentas were collected for immunohistochemistry.

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

Total RNA from placentas was extracted using TRI reagent [23]. RNase-free DNase-treated total RNA (1.0 mg) was reverse-transcribed with avian myeloid leukemia virus reverse transcriptase (Promega). Real-time RT-PCR was performed with a Light Cycler 480 SYBR Green I kit (Roche Diagnostics) using gene-specific primers as listed in Table 1. The comparative C_t-method was used to determine the amount of target, normalized to an endogenous reference (18*S*) and relative to a calibrator ($2-\Delta\Delta Ct$) using the Lightcycler 480 software (Roche, version 1.5.0).

Table 1
Primers for real-time RT-PCR.

Genes	Forward (5′-3′)	Reverse (5′-3′)
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Cyp24a1	ACTCGGAACGTCACCTCCTT	CCAGATGCACCGAGTCGAAG
Star	ATGGCCACACATTTTGGGGA	ACTGAGCAGCCAAGTGAGTT
Cyp11a1	AAGACCTGGAAGGACCATGC	CACCAGGGTACTGGCTGAAG
3βHsd	TTTTCAGCCACCACCATCTCA	GGTCTGTCCTTCCCAGTGATT
Cox2	GGGCTCAGCCAGGCAGCAAAT	GCACTGTGTTTGGGGTGGGCT
Tnfa	GATCGGTCCCCAAAGGGATG	CCACTTGGTGGTTTGTGAGTG
Il1β	CCCGCACTGAGGTCTTTCAT	GAGAAGTCAATAGCCAGGCAGA
Mcp1	GGCTGGAGAGCTACAAGAGG	GGTCAGCACAGACCTCTCTC
Mip2	GCTGTCCCTCAACGGAAGAA	CGAGGCACATCAGGTACGAT

2.4. Western blotting

For total and nuclear protein extraction, the methods were used on basis of our previous study [22]. For immunoblots, same amount of protein was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were incubated with antibodies, and then followed by signal development using an ECL detection kit. Relative quantification of each protein was calculated after normalization to loading control protein by densitometric analysis with Image-Pro Plus V7 software.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to determine the levels of prostaglandin F2α (PGF2α) in maternal serum by microplate assay (BIOTEK, SENERGY2, USA) according to manufacturer's protocol.

2.6. Serum 25(OH)D measurement

Serum 25-hydroxyvitamin D [25(OH)D] level was measured by Radioimmunoassay (RIA) (GC-911, Germany) [21]. The RIA kits, with ^{125}I labelled 25(OH)D as a tracer, were from Diasorin (DiaSorin Inc., Stillwater, MN, USA). The repeatability was determined through measuring the level of quality controls. The controls fell within the acceptable range given by the manufacturer. The detection range of 25(OH)D RIA kits is from 1.5 to 100 ng/ml. In the present study, all values fell within detection range.

2.7. Serum calcium and phosphorus measurement

Serum calcium and phosphorus were measured by routine laboratory methods using automatic biochemical analyzer (Roche, Modular DPP, NO. 1549–06).

2.8. Serum estradiol and progesterone measurement

Serum was separated by centrifugation and stored at −80 °C. Steroids were solubilized in PBS. The concentrations of serum estradiol and progesterone were measured by radioimmunoassay (GC-911, Germany) from Beijing north institute of biological technology (Beijing, China) according to the manufacturer's protocol.

Table 2
Preterm delivery rate (%) in four groups.

Groups	Time of delivery	No. of dams	Rate of preterm delivery
Control	GD18 1200 h to GD19 0800 h	20	0%
VD3	GD18 1200 h to GD19 0800 h	15	0%
LPS	GD15 1200 h to GD16 0800 h	10	50%
	GD16 0800 h to 1200 h	1	5%
	GD17 0800 h to GD18 0800 h	1	5%
	GD18 0800 h to GD19 0800 h	8	0%
	Total	20	60%*
LPS + VD3	GD15 1800 h to GD16 0800 h	2	10%
	GD16 0800 h to 1200 h	2	10%
	GD17 1800 h to GD18 0800 h	3	15%
	GD18 0800 h to GD19 0800 h	13	0%
	Total	20	35%†

Preterm delivery rate was analyzed using χ^2 analysis.
* $P < 0.05$ as compared with Control group.
† $P < 0.05$ as compared with LPS group.

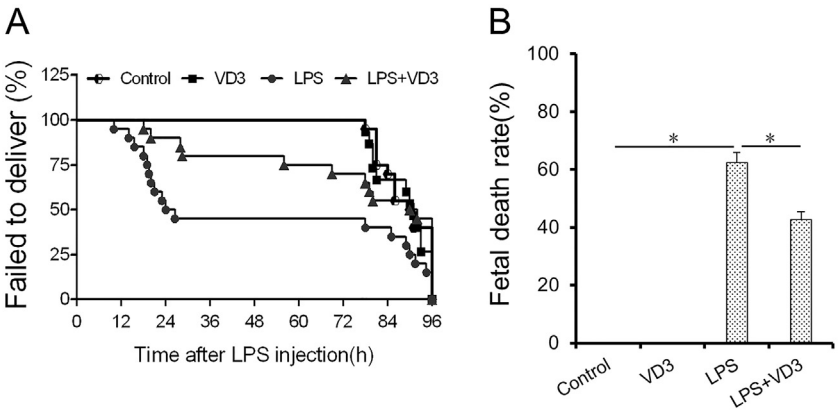


Fig. 1. Effects of oral VD3 supplementation on LPS-induced preterm delivery and fetal demise. Pregnant mice were administered with LPS or VD3 as in Materials and Methods. (A) All pregnant mice were observed for preterm delivery after LPS injection. (B) The mean fetal death rate per litter was assessed after delivery. The rates of preterm delivery and fetal death were analyzed using nonparametric tests (n = 15 for VD3 group and n = 20 for other groups). *$P < 0.05$.

Table 3
The level of serum calcium and phosphorus.

	Control	VD3	LPS	LPS + VD3
Calcium (mmol/L)	2.69 ± 0.061	2.71 ± 0.372	2.62 ± 0.054	2.66 ± 0.063
Phosphorus (mmol/L)	3.20 ± 0.231	3.49 ± 0.352	3.12 ± 0.164	3.15 ± 0.176

All data were expressed as means ± SEM. (n = 6). The level of serum calcium and phosphorus were performed using ANOVA and the Student–Newman–Keuls post hoc test. Values were not significantly different from each other ($P > 0.05$).

2.9. Immunohistochemistry

Placental sections were exhaustively sectioned at 5 μm thickness, deparaffinized, and rehydrated, then incubated for 20 min in 3% H_2O_2 to block endogenous peroxidases. After antigen retrieval and quenching of endogenous peroxidase, sections were incubated with COX-2, VDR or NF-κB p65 subunit monoclonal antibodies at 4 °C overnight, followed by a 30 min 37 °C incubation in goat anti-rabbit secondary. The color reaction was developed with HRP-linked polymer detection system. COX-2-positive cells and VDR/ NF-κB p65 subunit positive nucleus in the trophoblast were counted in twelve randomly selected fields from each slide at a magnification of ×400 [21].

2.10. Animal ethics

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 (Permit Number:13-0012). In this study, all mice were monitored at least twice per day. In addition, the rules of humane endpoints were strictly performed to determine when mice should be euthanized using a mixture of isoflurane and oxygen. All efforts were taken to minimize suffering when mice met our euthanasia criteria.

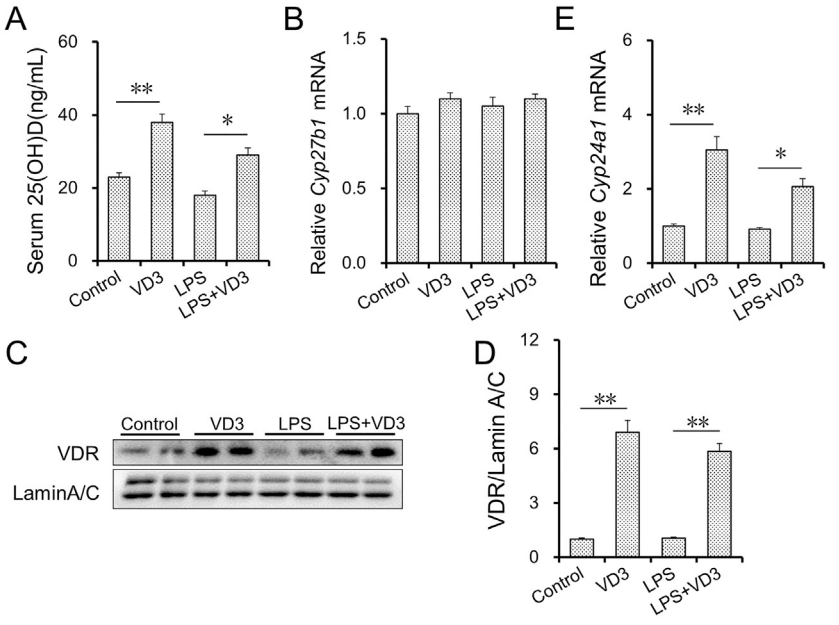
2.11. Statistical analysis

All statistical analyses were undertaken using SPSS 17.0. All quantitative data were expressed as mean ± standard error of the mean (SEM). Quantified data were expressed as means ± SEM. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups. The rates of preterm delivery and fetal death were analyzed using nonparametric tests. In all tests, statistically significance was defined at a level of $P < 0.05$.

3. Results

3.1. Oral VD3 supplementation alleviates LPS-induced preterm delivery and fetal demise

VD3 had no effect on rodent diet consumption and weight gain of pregnant mice (data not shown). No abortion was observed before LPS injection. No dam died throughout the pregnancy. In the Control and VD3 groups, no pregnant mice delivered before GD18. LPS treatment was quite effective in inducing preterm delivery (60%) (Table 2 and Fig. 1A, $P < 0.01$). Interestingly, the rate of preterm delivery dropped to 35% (7/20) in the LPS + VD3 group (Table 2 and Fig. 1A, $P < 0.01$). As shown in Fig. 1B, fetal death rate per litter (60.07%) was markedly increased in the LPS group as compared with Control group ($P < 0.01$). Interestingly, fetal death rate per litter was significantly



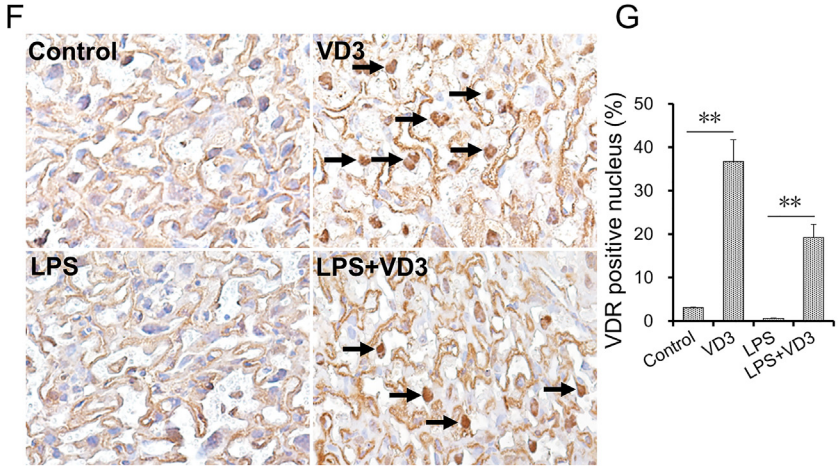


Fig. 2. Effects of oral VD3 supplementation on placental VDR signaling. Pregnant mice were administered with LPS or VD3 as in Materials and Methods. (A) Maternal sera were collected for 25(OH)D measurement using RIA. (B) Placental *Cyp27b1* mRNA was measured using real-time RT-PCR. (C) Placental nuclear VDR was measured using Western blotting. (D) Quantitative analysis of scanning densitometry at each time point was performed. (E) Placental *Cyp24a1* mRNA was measured using real-time RT-PCR. (F) Placental VDR was measured using immunohistochemistry, Scale bar: 50 μm. (G) Placental VDR positive nucleus were analyzed. Original magnification: 400×. Nuclear translocation of VDR was mainly distributed in mononuclear sinusoidal trophoblast giant cells of the labyrinth layer (black arrows). All data were expressed as means ± S.E.M. of six samples from six different pregnant mice. All experiments were repeated three times. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

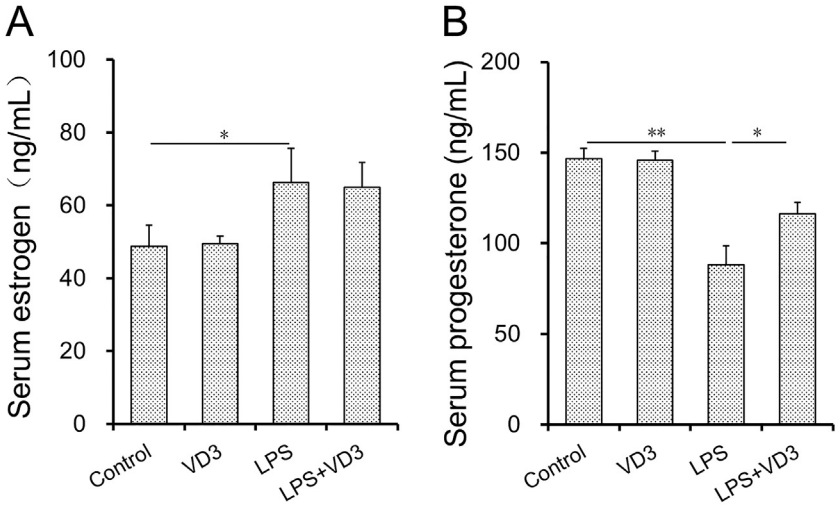


Fig. 3. Effects of oral VD3 supplementation on the levels of serum steroid hormones in LPS-treated pregnant mice. Pregnant mice were administered with LPS or VD3 as in Materials and Methods. (A) Maternal sera were collected for measurement of estradiol using RIA. (B) Maternal sera were collected for measurement of progesterone using RIA. All data were expressed as means ± S.E.M. of six samples from six different pregnant mice. All experiments were repeated three times. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

reduced when pregnant mice were orally administered with VD3 before LPS injection (Fig. 1B, $P < 0.05$).

3.2. Oral VD3 supplementation promotes placental VDR nuclear translocation

The levels of serum calcium and phosphorus were detected. As shown in Table 3, there were no significant differences on the levels of

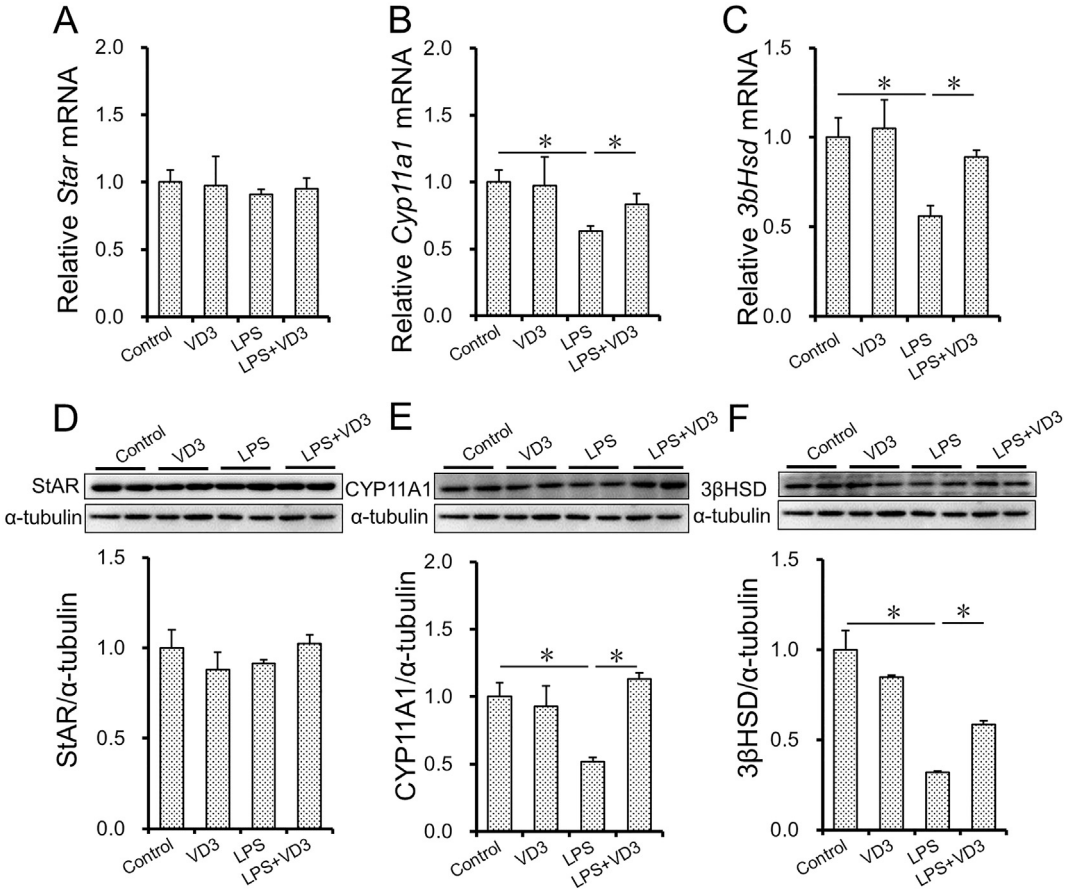


Fig. 4. Effects of oral VD3 supplementation on LPS-induced down-regulation of genes for progesterone biosynthetic enzymes in mouse placenta. Pregnant mice were administered with LPS or VD3 as in Materials and Methods. (A-C) The expression of placental genes for progesterone biosynthetic enzymes were determined using real-time RT-PCR. (A) *Star*; (B) *Cyp11a1*; (C) *3βHsd*. (D–F) The level of protein for progesterone biosynthetic enzymes in mouse placenta were measured using Western blotting. (D) StAR; (E) CYP11A1; (F) 3βHSD. All data were expressed as means ± S.E.M. of six samples from six different pregnant mice. All experiments were repeated three times. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups. *$P < 0.05$.

serum calcium and phosphorus among four groups. Serum 25(OH)D level were significantly increased in mice orally supplemented with VD3 (Fig. 2A, $P < 0.01$). No significant difference was observed on placental *Cyp27b1* mRNA, *a* hydroxylase of 25(OH)D_3, among four groups (Fig. 2B). Moreover, VDR nuclear translocation was elevated in placentas of mice with oral VD3 supplementation, VD3 supplementation improved placental VDR nuclear translocation (Fig. 2C and D, $P < 0.01$). Correspondingly, placental *Cyp24a1*, a downstream target gene of VDR, was up-regulated by VD3 supplementation (Fig. 2E, $P < 0.01$). Immunohistochemistry showed a strong VDR positive nucleus immunoreactivity in mononuclear sinusoidal trophoblast giant cells of the labyrinth layer (Fig. 2F). Interestingly, LPS obviously repressed VD3-induced nuclear translocation of VDR in trophoblast giant cells of the labyrinth zone (Fig. 2G).

3.3. Oral VD3 supplementation attenuates LPS-induced reduction of progesterone level in maternal serum

As expected, the level of estradiol in maternal serum was slightly elevated in LPS group (Fig. 3A, $P < 0.05$). By contrast, the level of progesterone in maternal serum was significantly alleviated in the LPS group (Fig. 3B, $P < 0.01$). Although VD3 had no effect on LPS-induced elevation of serum estradiol (Fig. 3A), significantly alleviated LPS-induced reduction of progesterone in maternal serum (Fig. 3B, $P < 0.05$).

3.4. Oral VD3 supplementation attenuates LPS-induced down-regulation of genes for progesterone biosynthetic enzymes in mouse placenta

No significant effects on placental *Star* mRNA and StAR protein were observed among four groups (Fig. 4A and D). As shown in Fig. 4B and E, the levels of placental *Cyp11a1* mRNA and CYP11A1 protein were significantly reduced when pregnant mice were treated with LPS ($P < 0.05$). Placental CYP11A1 were normalized in the LPS + VD3 group as compared with the LPS group (Fig. 4B and E, $P < 0.05$). As expected, the levels of placental *3βHsd* mRNA and 3βHSD protein were down-regulated in LPS-treated mice (Fig. 4C and F, $P < 0.05$). Interestingly, LPS-induced reductions on placental *3βHsd* mRNA and 3βHSD protein were significantly alleviated when pregnant mice were orally administered with VD3 before LPS injection (Fig. 4C and F, $P < 0.05$).

3.5. Oral VD3 supplementation attenuates LPS-induced up-regulation of estrogen receptor (ER)β in mouse placenta

Placental CYP19, a key enzyme for estradiol biosynthesis, was up-regulated in the LPS group (Fig. 5A and B, $P < 0.05$). In addition, the levels of placental ERα and ERβ were also elevated in the LPS group (Fig. 5A, C and D, $P < 0.01$). Oral VD3 supplementation had no effect on LPS-induced up-regulation of placental CYP19 and ERα (Fig. 5A–C, $P > 0.05$). Interestingly, oral VD3 supplementation attenuated LPS-induced up-regulation of ERβ in mouse placenta (Fig. 5A and D, $P < 0.05$).

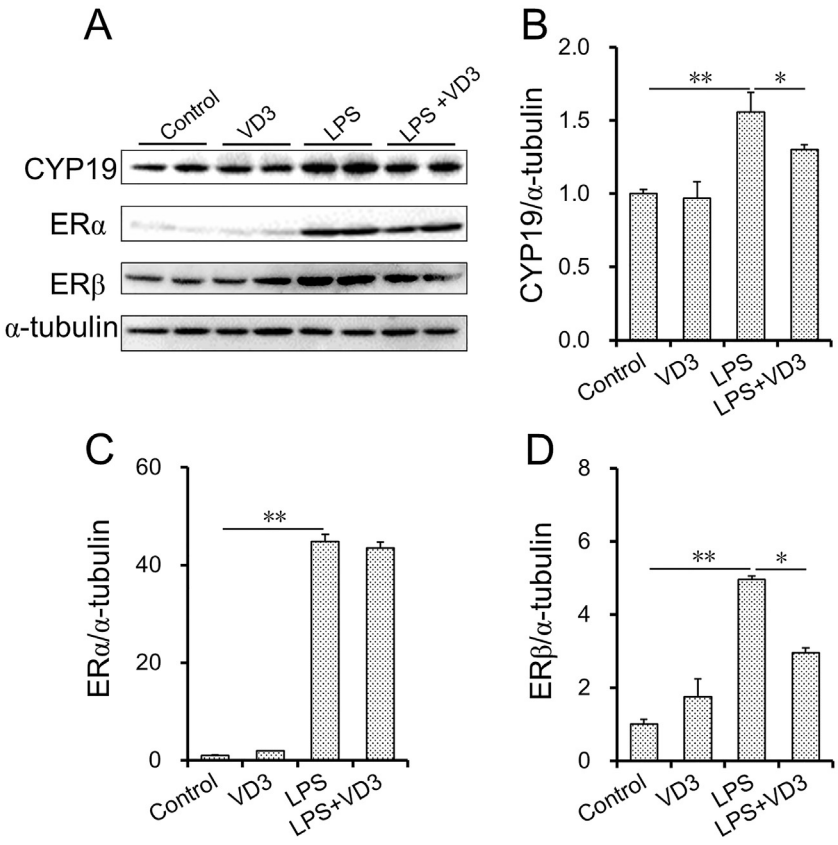


Fig. 5. Effects of oral VD3 supplementation on LPS-induced up-regulation of CYP19 and estrogen receptors in mouse placenta. Pregnant mice were administered with LPS or VD3 as in Materials and Methods. (A) Placental CYP19 and estrogen receptors were determined using Western blotting. (B–D) Quantitative analysis of scanning densitometry at each time point was performed. (B) CYP19; (C) ERα; (D) ERβ. All data were expressed as means ± S.E.M. of six samples from six different pregnant mice. All experiments were repeated three times. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.6. Oral VD3 supplementation attenuates LPS-induced placental COX-2 up-regulation and PGF2α increase in maternal serum

As expected, the level of PGF2α in maternal serum was significantly increased in the LPS group (Fig. 6A, $P < 0.01$). Interestingly, the level of PGF2α in the LPS + VD3 group was significantly decreased as compared with the LPS group (Fig. 6A, $P < 0.01$). Additionally, placental *Cox2* mRNA was up-regulated in the LPS group (Fig. 6B, $P < 0.05$). Moreover, placental COX-2 protein was also increased in the LPS group (Fig. 6C, $P < 0.05$). Immunohistochemistry showed a strong COX-2 immunoreactivity in placental trophoblast cells at 6 h after LPS injection (Fig. 6D and E, $P < 0.01$). Interestingly, LPS-induced up-regulation of placental COX-2 expression was attenuated when pregnant mice were oral supplementation with VD3 before LPS injection (Fig. 6B and C, $P < 0.01$; D and E, $P < 0.05$).

3.7. Oral VD3 supplementation inhibits LPS-evoked placental proinflammatory cytokines and chemokines

As shown in Fig. 7A and B, the levels of placental *Tnfα* and *Il1β* mRNAs, two proinflammatory genes, were significantly elevated in the LPS group ($P < 0.01$). In addition, the levels of placental *Mcp1* and *Mip2* mRNAs, two chemokine genes, were also significantly up-regulated in the LPS group (Fig. 7C and D, $P < 0.01$). Interestingly, oral VD3 supplementation alleviated LPS-induced up-regulation of placental *Tnfα*, *Il1β*, *Mcp1* and *Mip2* mRNAs (Fig. 7A–D, $P < 0.01$).

3.8. Oral VD3 supplementation inhibits LPS-induced placental NF-κB activation

As expected, the level of p-IκBα was significantly increased in the LPS group (Fig. 7E and F, $P < 0.01$). In the contrary, the level of p-IκBα was significantly decreased in the LPS + VD3 group as compared with the LPS group (Fig. 7E and F, $P < 0.01$). Correspondingly, the levels of nuclear NF-κB p50 and p65 subunits were obviously increased in the LPS group (Fig. 7E, G and H, $P < 0.01$), suggesting that maternal LPS exposure promotes translocation of placental NF-κB p65 and p50 subunits from cytoplasm to nucleus. As expected, oral VD3 supplementation significantly alleviated LPS-induced nuclear translocation of NF-κB p65 and p50 subunits in mouse placenta (Fig. 7E, G and H, $P < 0.05$). The effects of VD3 on LPS-induced nuclear translocation of NF-κB p65 subunit were further detected by IHC. As shown in Fig. 7I, LPS-induced nuclear translocation of NF-κB p65 subunit was mainly distributed in trophoblast giant cells of the labyrinth zone. As expected, VD3 pretreatment markedly blocked LPS-induced nuclear translocation of NF-κB p65 subunit in trophoblast giant cells of the labyrinth zone (Fig. 7J).

4. Discussion

In the present study, we aimed to investigate the effects of oral VD3 supplementation on LPS-induced preterm delivery and fetal demise. As expected, LPS treatment caused preterm delivery (60%) in ICR mice. Of interest, LPS-induced preterm delivery was attenuated when pregnant mice were oral VD3 supplementation before LPS injection. LPS-induced fetal demise was correspondingly alleviated by VD3. These results suggest that oral supplementation with VD3 significantly alleviates LPS-induced preterm delivery and fetal demise, which expands the preventive effects of VD3 against LPS-induced adverse pregnant outcomes.

Progesterone plays a critical role in the maintenance of myometrial quiescence throughout pregnancy [24]. In the early gestational stage, progesterone is synthesized by the corpus luteum [25]. In the late gestational stage, progesterone is produced by placenta to help maintain pregnancy [15]. CYP11A1 side chain cleavage enzyme (P450scc) is the rate-limiting enzyme that metabolizes cholesterol into pregnenolone [26]. Pregnenolone is then transformed into progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD) in placenta [27]. The present study found that placental CYP11A1 and 3βHSD were down-regulated

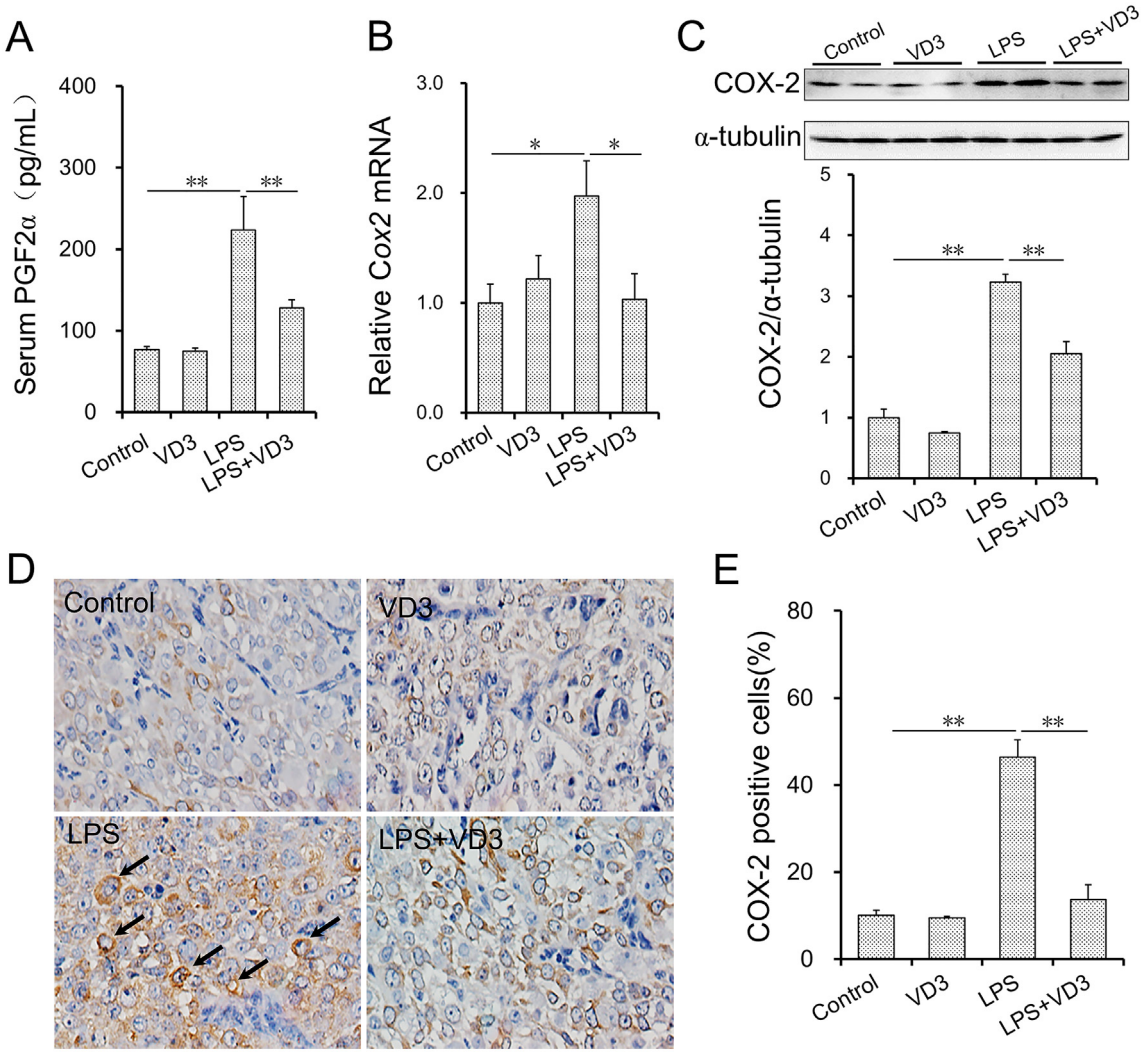


Fig. 6. Effects of oral VD3 supplementation on LPS-induced placental COX-2 up-regulation and PGF2α increase in maternal serum. Pregnant mice were administered with LPS or VD3 as in Materials and Methods. (A) Maternal sera were collected for measurement of PGF2α using ELISA. (B) Placental *Cox2* mRNA was measured using real-time RT-PCR. (C) Placental COX-2 protein was measured using Western blotting. (D) Placental COX-2 was measured using immunohistochemistry (arrow). (E) COX-2-positive cells were analyzed, Scale bar: 50 μm. All data were expressed as means ± S.E.M. of six samples from six different pregnant mice. All experiments were repeated three times. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

in the LPS group. Correspondingly, the level of progesterone in maternal serum was reduced in the LPS group. Of interest, oral supplementation with VD3 markedly attenuated LPS-induced reduction of progesterone in maternal serum. In addition, oral VD3 supplementation suppressed LPS-induced down-regulation of CYP11A1 and 3βHSD in mouse placenta. These results suggest that oral VD3 supplementation alleviates LPS-induced preterm delivery, at least partially, through regulating placental progesterone metabolism.

Several studies demonstrated that locally produced excess estrogens in the late gestational stage promoted parturition through stimulating genes for inflammatory cytokines and prostaglandins [28–30]. Indeed, estrogen receptors including ERα and ERβ were highly expressed in placentas of humans and other mammals [31,32]. The present study showed that placental CYP19, a key enzyme for estrogen synthesis, was obviously up-regulated in the LPS group. The level of estradiol in maternal serum was correspondingly elevated in the LPS group. In addition, both ERα and ERβ expression were up-regulated in placentas of LPS-treated mice. Although it had no effect on LPS-induced placental CYP19 up-regulation and excess estradiol production, oral VD3 supplementation partially reversed LPS-induced up-regulation of ERβ in mouse placenta. Thus, the present study does not exclude that oral VD3 supplementation decreases LPS-induced preterm delivery partially through regulating placental ER signaling.

Cyclooxygenase-2(COX-2) catalyzes the conversion of arachidonic acid to prostaglandin G2 and subsequently to prostaglandin H_2, which acts as a substrate for multiple isomerases that are individually responsible for the generation of eicosanoid products, including prostaglandin E_2, prostacyclin, and thromboxane A_2. Several studies demonstrated that excess prostaglandins were involved in the process of preterm delivery [33,34]. The present study showed that placental COX-2 expression was up-regulated in the LPS group. The concentration of PGF2α in maternal serum was correspondingly elevated in LPS-treated mice. Recently, an in vitro report found that 1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$], the active form of VD3, suppressed LPS-induced upregulation of COX-2 in macrophage [35]. The present study investigated the effects of oral VD3 supplementation on LPS-induced placental COX-2 upregulation and excess PGF2α production. Of interest, oral supplementation VD3 attenuated LPS-induced placental COX-2 up-regulation. In addition, LPS-evoked excess PGF2α production was alleviated in VD3-administered mice. These results suggest that oral VD3 supplementation alleviates LPS-induced preterm delivery partially through regulating placental prostaglandin metabolism.

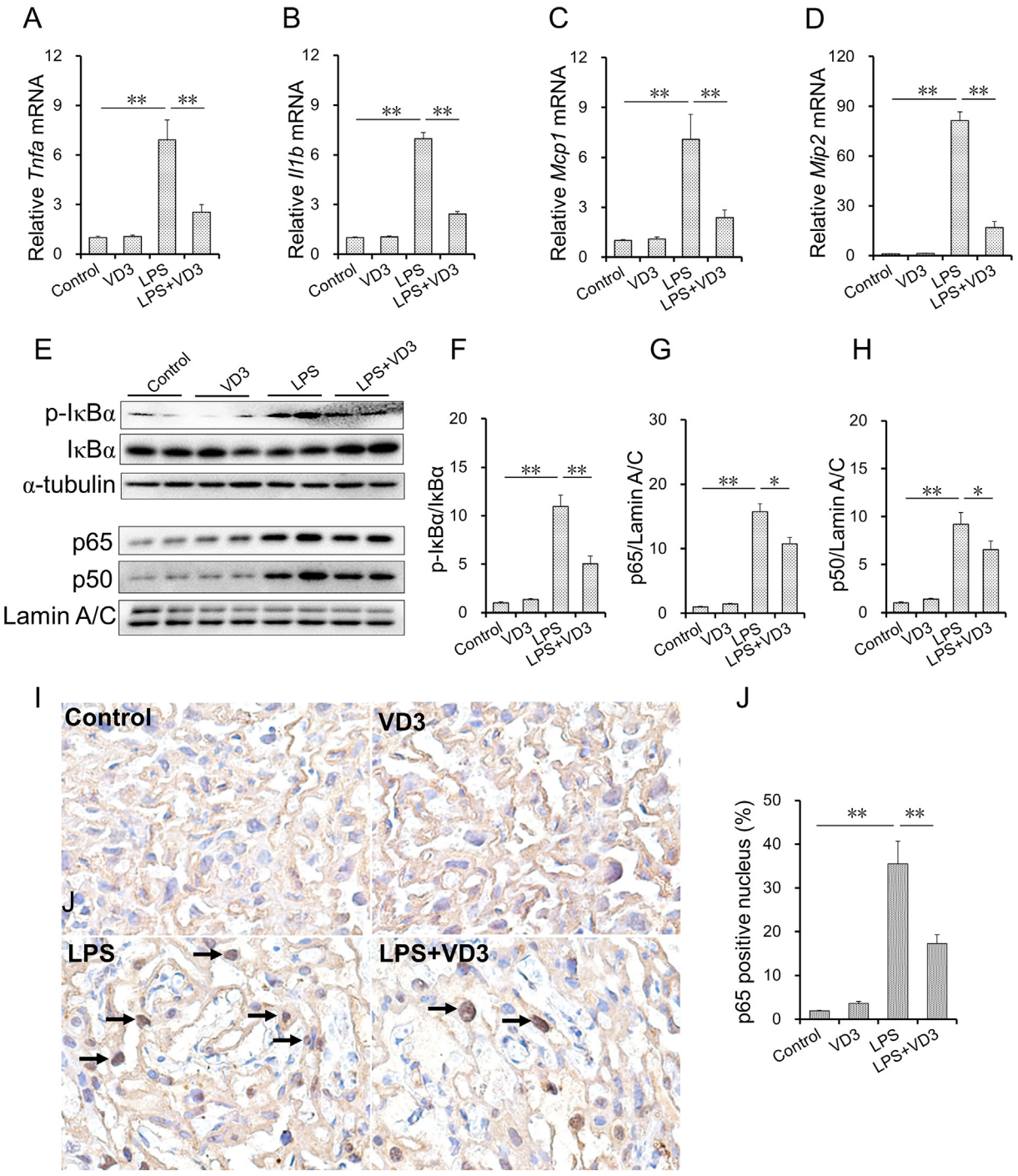


Fig. 7. Oral VD3 supplementation inhibits LPS-evoked placental proinflammatory cytokines and chemokines and placental NF-κB activation. Pregnant mice were administered with LPS or VD3 as in Materials and Methods. (A-D) Placental *Tnfα, Il1β, Mcp1* and *Mip2* mRNAs were measured using real-time RT-PCR. (A) *Tnfα;* (B) *Il1β;* (C) *Mcp1;* (D) *Mip2.* (E) Placental p-IκBα, IκBα, NF-κB p65 and NF-κB p50 were determined using Western blotting. (F–H) Quantitative analysis of scanning densitometry at each time point was performed. (F) p-IκBα and IκBα. (G) NF-κB p65. (H) NF-κB p50. (I) Placental NF-κB p65 subunit was measured using immunohistochemistry, Scale bar: 50 μm. Original magnification: 400×. Nuclear translocation of NF-κB p65 subunit was mainly distributed in trophoblast giant cells of the labyrinth layer (black arrows). (J) Placental NF-κB p65 positive nucleus were analyzed. All data were expressed as means ± S.E.M. of six samples from six different pregnant mice. All experiments were repeated three times. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

Numerous studies indicated that proinflammatory cytokines and chemokines are involved in LPS-induced preterm delivery in rodent animals [36,37]. Indeed, VD3 has an anti-inflammatory activity [38]. The present study showed that the levels of placental *Tnfα* and *Il1β* mRNAs, two proinflammatory genes, were significantly elevated in the LPS group. Correspondingly, *Mcp1* and *Mip2* mRNAs, two chemokine genes, were significantly elevated in LPS-treated mice placentas. VD3 pretreatment attenuated LPS-evoked elevations of proinflammatory and chemokines mRNAs in the placentas. These results suggest that oral supplementation VD3 decreases LPS-induced preterm delivery partially through its anti-inflammatory activity.

A recent report from our laboratory found that VD3 had no effect on

placental *tlr*4 mRNA. Besides, VD3 had little effect on LPS-induced up-regulation of placental MyD88 protein. These results demonstrated that VD3 did not inhibit directly LPS binding to its receptors or down-regulate LPS sensors. According to our previous report, VD3 had no effect on LPS-evoked placental p38 MAPK and ERK1/2 phosphorylation. Moreover, VD3 pretreatment did not inhibit LPS-elevated Akt phosphorylation. In addition, VD3 pretreatment did not block LPS-elevated IκBα phosphorylation at 2 h after LPS injection [38]. In the present study, we found that VD3 pretreatment dramatically repressed LPS-induced placental IκBα phosphorylation at 6 h after LPS injection. In addition, VD3 pretreatment remarkably suppressed LPS-induced nuclear translocation of NF-κB p65 and p50 subunits in mouse placenta. These results indicate that VD3 inhibits LPS-induced inflammatory cytokines partially through suppressing placental NF-κB activation.

The present study found that nuclear translocation of VDR was observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth layer through immunohistochemistry. Correspondingly, nuclear translocation of NF-κB p65 subunit was also observed in mononuclear sinusoidal trophoblast giant cells of the labyrinth layer. Moreover, several reports showed that VD3 inhibited NF-κB signaling through reinforcing the interaction between VDR and NF-κB p65 subunit in mouse placentas and kidneys [38,39]. Above results indicate that VD3 inhibits LPS-induced inflammation partially through inhibiting placental NF-κB signaling in VDR-dependent manner. Thereby, we conclude that cholecalciferol suppresses proinflammatory cytokines and chemokines through directly blocking NF-κB p65, thus alleviating LPS-induced preterm delivery.

CYP24a1 is a downstream target gene of VDR and also a degradative enzyme gene of 25-hydroxycholecalciferol [25(OH)D_3] and 1,25$(OH)_2D_3$. CYP24a1 is able to modulate circulating concentrations of 1,25$(OH)_2D_3$, resulting in an appropriate cellular response [40]. CYP27b1 is a hydroxylase of 25(OH)D_3, which metabolizes 25(OH)D_3 to 1,25$(OH)_2D_3$, the active form of vitamin D [41]. The present study investigates the effects of VD3 on *Cyp27b1* and *Cyp24a1* mRNAs in the placenta. Our results demonstrated that no significant difference was observed on placental *Cyp27b1* mRNA among four groups. In contrast, placental *Cyp24a1* mRNA was up-regulated in VD3-treated mice. These findings indicate that supplemental VD3 had no effect on anabolic enzyme expression, but up-regulated catabolic enzyme expression.

The results from the present study may have clinic implications. Increasing evidence has demonstrated that vitamin D insufficiency/deficiency is common in pregnant women [19,20,42–44]. A recent report from rodent animals found that gestational oral VD3 supplementation alleviated LPS-induced neural tube defects [45]. Moreover, gestational oral VD3 supplementation attenuated LPS-induced early embryo loss and fetal growth restriction in mice [21,34]. The present study found that oral VD3 supplementation decreased LPS-induced preterm delivery and fetal demise. Thus, VD3 may be used as a protective agent especially in high-risk situations when pregnant women are infected with bacteria. A multicenter randomized controlled trial is necessary to determine whether oral VD3 supplementation alleviated adverse pregnant outcomes.

In summary, the present study investigated the effects of oral VD3 supplementation on LPS-induced preterm delivery and fetal demise. We found that oral VD3 supplementation alleviated LPS-induced preterm delivery and fetal demise. Moreover, oral VD3 supplementation blocked LPS-evoked reduction of serum progesterone through down-regulating placental progesterone synthetic enzymes. In addition, oral VD3 supplementation suppressed LPS-induced production of placental prostaglandins. These results provide evidence that VDR-mediated alleviation on LPS-induced preterm delivery and fetal demise might be, at least partially, attributed to its regulation of placental steroid hormones and prostaglandins.

Author contributions

Participated in research design: De-Xiang Xu

Conducted experiments: Lin Fu, Shen Xu, Zhen Yu, Zhi-Hui Zhang, Cheng Zhang, Hua Wang and Yuan-Hua Chen.

Contributed new reagents/materials/analysis tools: Yuan-Hua Chen and De-Xiang Xu

Performed data analysis: Lin Fu

Wrote or contributed to the writing of the manuscript: De-Xiang Xu

All authors read and approved the final manuscript.

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Competing financial interests

The authors report no conflict of interests.

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