RESEARCH ARTICLE

Therapeutic effect and mechanism of different doses of aspirin on preterm delivery in pregnant mice

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Abstract

Background

Preterm birth is a major cause of perinatal mortality and complications, with inflammation being a key contributing factor. Current treatments, like uterine contraction inhibitors and antibiotics, are unsatisfactory. Aspirin, a cyclooxygenase inhibitor, shows promise in treating infectious preterm labor but has limited in vivo studies and an unclear mechanism.

Methods

In this study, a mouse model of infectious preterm birth was established via lipopolysaccharide (LPS) injection, and the aspirin doses used in these animals were converted from the recommended human doses by the body surface area method, with the high-dose and low-dose groups set at 0.78 mg/kg and 0.21 mg/kg, respectively. ELISA detected inflammatory factors TNF-α, IL-1β, IL-6 in serum, amniotic fluid and placenta. WST-8 kit and TBA method measured serum SOD activity and MDA content, respectively. DTNB colorimetric method analyzed glutathione content in liver and placenta. Western blot detected MyD88, IκB, p-IκB and nucleus NF-κB p65 protein expression in uterine tissues.

Results

Results showed the 75 μg/kg LPS group had a 91.7% preterm birth rate and 4.67% stillbirth rate. Low-dose (66.7%) and high-dose (41.6%) aspirin reduced preterm birth and increased live birth rates, with significant intergroup differences ($P < 0.05$). Aspirin lowered LPS-induced TNF-α, IL-1β, IL-6 in serum, amniotic fluid and placenta, regulated oxidative stress, reversed MyD88/p-IκB overexpression and reduced p65 nuclear translocation. TLR4/NF-κB inhibitors downregulated these factors and nuclear NF-κB p65/p-IκB. Additionally, we found that inflammatory LPS induced

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abnormal fetal mouse skeletal development (e.g., malformation and deficiency), which was ameliorated by aspirin exposure.

Conclusion

Aspirin may ameliorate preterm birth by up-regulating TLR4/NF-κB pathway, laying theoretical basis for aspirin clinical application in preterm birth.

1. Introduction

Preterm birth refers to delivery before 37 weeks of gestation and is one of the leading causes of neonatal mortality and morbidity worldwide, posing a serious challenge to public health and social development [1–4]. Epidemiological data show that approximately 11% of newborns globally are preterm, and complications related to preterm birth account for nearly one-third of infant deaths [5,6]. These infants not only face a high risk of mortality in the neonatal period but are also prone to various short-term complications such as respiratory distress syndrome, periventricular leukomalacia, necrotizing enterocolitis, and retinopathy of prematurity [7,8]. Long-term effects cannot be ignored, including neurocognitive impairments, decreased visuospatial abilities, and increased risk of adult cardiovascular metabolic diseases. Additionally, the socioeconomic burden of preterm birth is enormous, involving direct medical costs and long-term family and social support needs [9–11]. Although current clinical strategies employ various treatments (such as tocolytics, corticosteroids for fetal lung maturation, and antibiotics for infection), their effectiveness is limited, and the rate of preterm birth remains high [3,12–16]. The root cause lies in the insufficient understanding of the mechanisms of preterm birth and the lack of effective prevention and treatment methods.

The mechanisms of preterm birth are complex, with inflammation considered a common core pathological pathway [17,18]. Whether infectious (e.g., microbial invasion) or sterile inflammation (e.g., stress or ischemia-reperfusion injury), it can activate inflammatory responses at the maternal-fetal interface, induce uterine contractions and cervical maturation, ultimately leading to premature delivery [19,20]. This process involves the regulation of multiple signaling pathways, including classic inflammatory pathways such as NF-κB, p38 MAPK, and cGAS-STING. Activated pathways promote the release of pro-inflammatory cytokines (e.g., IL-6, IL-8, TNF-α), triggering local and systemic inflammatory responses that affect cervical remodeling, amniotic membrane integrity, and myometrial contractility [21]. Among these, the NF-κB pathway, as a central regulator of inflammatory responses, is believed to be a key molecular event in the occurrence of preterm birth when abnormally activated [22–25].

In the classic model of preterm birth, lipopolysaccharide (LPS), as a natural ligand of Toll-like receptor 4 (TLR4), initiates intracellular signaling by binding to this receptor. NF-κB is a key transcription factor in the TLR4 signaling pathway [26–28]. Under resting conditions, NF-κB binds to its inhibitory protein IκB, forming a complex that

remains sequestered in the cytoplasm [29]. Upon inflammatory stimulation such as LPS exposure, IκB undergoes phosphorylation and degradation, allowing NF-κB to be released and translocate into the nucleus [30,31]. The activated NF-κB subsequently initiates the transcriptional expression of various pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-8. This cascade of molecular events ultimately drives uterine inflammatory responses and the initiation of contractions, thereby contributing to the onset and progression of preterm birth [32].

Aspirin (Asp) is a significant non-steroidal anti-inflammatory medication (NSAID), primarily controlling the inflammatory response by inhibiting cyclooxygenase (COX) and subsequently reducing the production of prostaglandin E2 [33–36]. It is a key component in the treatment of many diseases and has a wide range of pharmacological effects, including antipyretic, analgesic, anti-inflammatory, anti-platelet, and thrombosis inhibition [36–38]. Additionally, aspirin has antioxidant properties and can react with hydroxyl radicals (•OH), superoxide anion radicals $O_2^{\bullet-}$, and H_2O_2 to protect cells and inhibit aging [39–41]. In 2022, WHO recommended the use of low-dose aspirin (75 mg/ day) for the prevention of preeclampsia in women with moderate to high-risk pregnancies. Low-dose aspirin (81 mg/day) beginning between 6 weeks and 0 days of gestation and between 13 weeks and 6 days of gestation decreased the risk of preterm birth before 37 weeks and decreased perinatal death, according to a randomized, multi-country, double-blind, placebo-controlled experiment [40–43]. In recent years, with in-depth research into the inflammatory mechanisms of pregnancy complications such as preeclampsia, aspirin has been proposed as a potential agent for preventing preterm birth [41,44]. Multiple clinical studies have shown that low-dose aspirin can reduce the incidence of preterm birth in high-risk populations [39–41]. For example, a Swedish register-based cohort study of women with a previous preterm birth indicated that low-dose aspirin use was associated with a reduced risk of recurrent preterm birth, particularly a significant reduction in spontaneous preterm birth [41]. The Aspirin trial demonstrated that in nulliparous women without additional risk factors, low-dose aspirin significantly reduced the risk of spontaneous preterm birth before 34 weeks. Additionally, the EAGeR trial suggested that preconception initiation of low-dose aspirin may show beneficial trends in preventing preterm birth by improving placental perfusion and suppressing inflammation [45]. However, the specific mechanisms, particularly whether it mediates anti-inflammatory effects through regulation of the NF-κB pathway, remain unclear. Existing basic research indicates that aspirin can inhibit the activation of NF-κB and the expression of downstream inflammatory factors, but experimental evidence for this effect in the context of preterm birth is still relatively lacking.

Therefore, this study aims to investigate the effects of aspirin intervention on key inflammatory cytokine levels and NF-κB signaling pathway activation by constructing a lipopolysaccharide (LPS)-induced inflammatory preterm birth mouse model [46–48], with the goal of revealing the potential molecular mechanisms by which aspirin prevents preterm birth and providing a theoretical basis for clinical intervention.

2. Materials and methods

2.1 Materials

Bacterial lipopolysaccharide (LPS, *Escherichia coli*, 055:B5) was purchased from Solarbio. Aspirin, carbol fuchsin and potassium hydroxide were purchased from Sinopharm Group Reagent (Shanghai, China). TAK-242 (Resatorvid, CAS No.: 243984-11-4) with a purity of ≥99% was supplied by Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). BAY 11−7082 (CAS No.: 19542-67-7) was purchased from Proteintech Group (Wuhan Sanying Bio-Technology Co., Ltd., Wuhan, China) and used without further purification. TNF-α (PT512), IL-1β (PI301), IL-6 (PI326) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Biyuntian Biotechnology. TNF-α, IL-1β and IL-6 specific primers were synthesized by Shanghai Shenggong Bioengineering Technology Service Co., LTD. The assay kits for superoxide dismutase (SOD) activity (catalog No.: S0101S) and reduced glutathione (GSH) content (catalog No.: S0053) were both purchased from Beyotime Biotechnology (Shanghai, China). The malondialdehyde (MDA) assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MyD88, p-Iκb, Iκb, NF-κB p65, β-actin and Histone H3 antibody, HRP labeled goat anti-rabbit and Goat anti-mouse secondary antibody purchased from Proteintech

(Wuhan, China). In all experiments, deionized water (Millipore Milli-Q grade) with a resistivity of 18.2 MΩ was utilized. Water treated with DEPC was used to prepare all of the solutions. Other reagents and pharmaceuticals are high quality analytical pure commodities.

2.2 Animal sources and modeling

Since inflammation needs to be simulated in live pregnant animals, and cannot be replaced by cell cultures or non-pregnant animal models, a pregnant mouse model must be used. Specific-pathogen-free (SPF) KM mice (20 males and 180 females), aged 9–10 weeks with body weights of 34–36 g for males and 28–30 g for females, were purchased from Shanghai Bikai Keyi Biotechnology Co., Ltd. (License No.: SCXK (Shanghai) 2018–0006). The mice were housed in standard polycarbonate cages (30 × 18 × 15 cm) with 3–5 mice per cage (same-sex grouping). The cages were lined with autoclaved corncob bedding and environmental enrichment was provided by adding sterile wooden gnawing sticks and plastic shelters. The animals were fed a standard commercial rodent diet (Product No.: 10400R, Beijing Huafukang Biotechnology Co., Ltd.) ad libitum and had free access to high-pressure sterilized water. Per GB 14922.2–2023 (national standard for laboratory animal quality control), these mice are serologically negative for core pathogens that interfere with experiments or impair health, including murine hepatitis virus, Sendai virus, Salmonella spp., Pasteurella pneumotropica, Toxoplasma gondii, and ectoparasites (e.g., mites). All animal experimentation complied with the ARRIVE guidelines and was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (document No. 2023113). All experiments were performed in accordance with relevant guidelines and regulations. Pre-experiment adaptive feeding 1 week (free diet, day and night equilibrium, temperature 20–25 °C, humidity 50 ± 5%). For mating, male and female mice were caged at a ratio of 2:4 (♂:♀) at 9:00 PM, and the female mice were examined at 7:00 AM the next morning. The day on which a vaginal plug was detected was designated as gestational day 0 (GD 0). The behavioral parameters (including normal movement ability, sleepiness or not, etc.) and health conditions (hair quality, food and water intake, etc.) of the pregnant mice were monitored separately in the morning and evening every day. On gestational day 7 (GD 7), transabdominal ultrasound was performed to confirm pregnancy. After corresponding group experiments, on the 18th day, relevant data were statistically analyzed. Euthanasia was performed per the Guidelines for the Care and Use of Laboratory Animals. Pregnant mice were anesthetized by exposing to CO_2 (10–15 L/min) in a closed chamber until unconscious (no paw pinch response), followed by 5 additional minutes of CO_2 exposure to ensure complete euthanasia. Uterine horns were then rapidly dissected to collect fetuses, which were immediately euthanized by immersing in liquid nitrogen (−196 °C) for 30 seconds-a validated method for instantaneous fetal rodent death. Vital signs were assessed 2 minutes post-euthanasia to confirm death: pregnant mice were confirmed dead by absent heartbeat (abdominal palpation), no chest movement, and loss of corneal reflex; fetuses by no limb twitching, undetectable heartbeat (thoracic inspection), and pale, non-pulsatile umbilical vessels.

2.3 Therapeutic effect of aspirin on LPS-induced premature birth

The exposure dose of aspirin used in this study was derived from the human exposure dose, which was converted to the mouse dose based on the body surface area (BSA) normalization method. The relationship between BSA and body weight (BW) follows the formula: $S = kW^{2/3}$, where S represents BSA (m^2), W denotes BW (kg), and k is a species-specific constant (k = 0.1 for humans and k = 0.06 for mice). Based on the calculations, we selected 0.21 mg/kg and 0.78 mg/kg as the low and high doses for the study, respectively.

To observe the effects of aspirin supplementation on adverse pregnancy outcomes caused by exposure to low-dose LPS during the second trimester, pregnant mice were randomly divided into 6 groups with 15–18 mice in each group, as follows: (1) Blank control group: subcutaneous injection of the same amount of normal saline, GD15 intraperitoneal injection of the same volume of normal saline. (2) LPS control group: subcutaneous injection of the same amount of normal saline, GD15 intraperitoneally injected LPS 75 μg/kg. (3) High-dose aspirin control group: The same amount of normal

saline was injected intraperitoneally on GD15, and 0.78 mg/kg aspirin was injected half an hour later. (4) Low-dose aspirin control group: The same amount of normal saline was injected intraperitoneally on GD15, and 0.21 mg/kg aspirin was injected half an hour later. (5) High-dose aspirin treatment group: GD15 intraperitoneal injection of LPS 75 μg/kg, half an hour later, 0.78 mg/kg aspirin was injected. (6) Low-dose aspirin treatment group: GD15 intraperitoneal injection of LPS 75 μg/kg, half an hour later, 0.21 mg/kg aspirin was injected. After dosing on gestational day 15 (GD15), all pregnant mice were continuously monitored for 72 hours (i.e., until GD18) for birthing signs—a time window determined based on the physiological characteristics of mouse pregnancy (normal gestation period: 19–21 days) and the known latency of LPS-induced preterm birth in this model. The number of premature births, stillbirths, and live births was recorded.

2.4 Skeletal and malformation analysis of fetal mice

On gestational day 18 (GD18), pregnant mice were euthanized, and fetal mice were harvested from the uterus. The fetal mice were then skinned, with muscles and internal organs removed, followed by immersion in alizarin red staining solution (composed of alizarin red and potassium hydroxide) for 1 week. After staining, the solution was discarded, residual corroded muscles were carefully picked off, and the bones were transferred to clearing solution A (containing glycerin and potassium hydroxide) for 2 days. Finally, the bones were transferred to 50% glycerin and stored for examination.

2.5 Effects of aspirin on inflammation and oxidative stress induced by LPS in the second trimester

To investigate the effects of aspirin supplementation on inflammation and oxidative stress induced by low-dose LPS exposure during the second trimester, 60 pregnant mice were randomly divided into the following 10 groups, namely: Control group, LPS group, high-dose aspirin control group, low-dose aspirin control group, LPS+high-dose aspirin group, LPS + low-dose aspirin group, TAK-242 inhibitor (TRL4 inhibitor) group, BAY 11–7082 inhibitor (NF-κB inhibitor) group, LPS+High-dose aspirin + TAK-242 inhibitor group and LPS+High-dose aspirin + BAY 11–7082 inhibitor group. On gestational day 15 (GD15), all pregnant mice were euthanized via cervical dislocation under sterile conditions 4 h after LPS treatment. Serum was collected by cardiac puncture and centrifuged at 3000 × g for 15 min at 4 ℃ to separate the supernatant; amniotic fluid was aspirated from amniotic sacs after careful uterine incision. Both samples were aliquoted into RNase/DNase-free EP tubes with group labels, snap-frozen in liquid nitrogen, and stored at −80 ℃ for subsequent ELISA detection. The liver and placenta of pregnant mice were frozen in liquid nitrogen and stored at −80 ℃ for real-time quantitative RT-qPCR, Western blotting, and reduced GSH detection.

2.6 Elisa analyzed the inflammatory components in serum and amniotic fluid

ELISA was used to quantify TNF-α, IL-1β, and IL-6 in serum and amniotic fluid of pregnant mice. The kit was equilibrated to room temperature for 20 min. Eight standard wells were prepared for standard curve establishment. 100 μL samples were added to each well, followed by incubation at 37 ℃ for 120 min. After 5 washes with washing buffer (blot-dried), 100 μL biotinylated antibody was added per well, sealed with a transparent plate sealer, and incubated at room temperature for 60 min. Post re-washing, 100 μL horseradish peroxidase-conjugated streptavidin was added, sealed with a white plate sealer, and incubated in the dark at room temperature for 20 min. 50 μL stop solution was added and mixed gently. OD values were measured at 450 nm immediately via microplate reader, and cytokine concentrations were calculated using OD values and standard curves.

2.7 Determination of Malondialdehyde (MDA) and superoxide dismutase (SOD) content

SOD activity was determined by the WST-8 method: WST-8 reacts with superoxide anions (O_2^-) generated by xanthine oxidase (XO) to form water-soluble formazan, and superoxide dismutase (SOD) inhibits this reaction via catalyzing O_2^- dismutation, resulting in a negative correlation between SOD activity and formazan production. SOD activity was quantified

by colorimetric analysis of the reaction product. MDA was quantified via the thiobarbituric acid (TBA) method: MDA condenses with TBA under acidic and high-temperature conditions to form a TBA adduct, which is detected by measuring fluorescence intensity. Liver tissues from pregnant mice were homogenized on ice, centrifuged at 12,000 × g and 4°C for 5 min, and the supernatant was used as the test sample. Protein concentration was determined using a BCA protein assay kit.

2.8 Determination of reduced glutathione content

The content of glutathione (GSH) in placental tissue was determined by the DTNB method: DTNB (5,5'-dithiobis-2-nitrobenzoic acid) reacts with reduced glutathione (GSH) to form a yellow-colored 5-thio-2-nitrobenzoic acid (TNB), with the absorbance of TNB being proportional to GSH concentration. GSH content was quantified by colorimetric analysis of the reaction product. Briefly, a GSH standard curve was established first. For sample preparation, 100 mg of placental tissue from each mouse was homogenized in 1 mL of pre-cooled (4 °C) 1 × GSH extraction buffer on ice, followed by centrifugation at 12,000 rpm for 20 min at 4 °C. The collected supernatant was used for GSH analysis, and its volume was recorded. The reaction between GSH in the supernatant and DTNB generated stable yellow TNB, and the absorbance at 412 nm was measured by spectrophotometry. GSH content in the samples was calculated by referencing the standard curve.

2.9 RT-qPCR analysis

About 50 mg of placental tissue was taken from each mouse, and 1 mL of TRIzol Reagent was added to extract total RNA. The absorbance value of total RNA of the sample was determined by spectrophotometer at 260 nm wavelength, and the total RNA content of the sample was calculated, and the total RNA of the sample was quantified to 0.5 μg/μL. In each sample, 2.0 μg of total RNA was used for cDNA synthesis, and the remaining genomic DNA in total RNA was digested by DNase without RNase. The FastKing RT Kit (With gDNAse) was used to reverse-transcribe the mRNA. The RT-qPCR was performed using SYBR premix Ex Taq TM (Takara). The $2^{-\triangle\triangle Ct}$ method was employed for data analysis. All experiments were repeated at least three times. The primer sequences are shown in Table 1.

2.10 Western blotting analysis

The placental tissue was homogenized in RIPA lysate supplemented with protease and phosphatase inhibitors, placed on ice for 20 min, and centrifuged at 14,000 r/min for 20 min at 4 °C. The supernatant was collected, and protein concentration was quantified using the BCA Protein Assay Kit with a bovine serum albumin (BSA) standard curve (absorbance measured at 562 nm). Equal protein amounts (e.g., 30 μg per lane) were adjusted by adding loading buffer, and protein quality was verified by Coomassie blue staining. Subsequent steps included protein electrophoresis, membrane transfer, blocking with 5% fetal bovine serum for 30 min at room temperature, overnight incubation with primary antibodies (MyD88, p-IκB, β-Actin rabbit antibodies) in 5% fetal bovine serum, 3 × 10 min TBST washes, 1 h room temperature incubation with HRP-conjugated secondary anti-rabbit antibody, chemiluminescent visualization, and quantitative analysis of target band optical density using ImageJ software.

Table 1. Primers for RT-qPCR in our study.

Gene	Sense primer	Antisense primer
Mouse TNFα	ACTCCAGGCGGTGCCTATGT	GTGAGGGTCTGGGCCATAGAA
Mouse IL-1β	GCCTCGTGCTGTCGGACCCATAT	TCCTTTGAGGCCCAAGGCCACA
Mouse IL-6	AGACAAAGCCAGAGTCCTTCAGAGA	GCCACTCCTTCTGTGACTCCAGC
GAPDH	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG

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2.11 Statistical analysis

All analyses were performed using SPSS 26.0 (IBM) and GraphPad Prism 9.0 under the guidance of a biostatistician, with a two-tailed $P < 0.05$ considered statistically significant. The sample size was determined via G*Power 3.1 software, with the significance level (α) set to 0.05 and statistical power (1-β) to 0.8 (a universally accepted threshold in clinical and experimental research). Based on the preterm birth rate data from previous similar studies [49,50]—>90% in the LPS-induced preterm birth model (treatment group, P_1) and ≤10% in the normal control group (P_2) with an allocation ratio of 1 — the required sample size was calculated to be 7 animals per group. To account for potential experimental losses (e.g., accidental death), 12 animals were ultimately included per group, exceeding the minimum required sample size. Normality of continuous data was verified using the Shapiro-Wilk test ($P > 0.05$), and homogeneity of variance was confirmed via Levene's test. Continuous variables were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test for post-hoc pairwise comparisons. Categorical data (Table 2) used Pearson's chi-square test, with χ^2 and p values reported. Effect sizes were included where relevant. All results are presented as mean ± variability (or median with interquartile range for non-normal data), with *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ considered statistically significant.

3. Results

3.1 Therapeutic effect of different doses of aspirin on premature delivery induced by LPS in mice

Inflammation is the most common cause of preterm birth, and domestic and foreign studies have induced preterm birth in mice by intraperitoneal injection of LPS. The adverse pregnancy outcomes caused by exposure to low-dose LPS during the second trimester of mice are shown in Table 2 and Fig 1. Compared with the sham group, intraperitoneal injection of LPS at 75 μg/kg significantly induced preterm birth in mice, with an incidence of 91.7%. Neither high-dose nor low-dose aspirin induced preterm birth. Compared with the preterm birth rate of 91.7% in the LPS group, the incidences of preterm birth in the 0.21 mg/kg and 0.78 mg/kg aspirin treatment groups were 66.7% and 41.7%, respectively, with statistically significant differences. According to the bone map, the main types of skeletal malformations induced by LPS exposure were occipital dysplasia, rib malformation, and sternal malformation. Intraperitoneal injection of 0.21 mg/kg and 0.78 mg/kg aspirin significantly reduced LPS-induced rib and sternal malformations in fetal mice (S1 Fig in S1 File).

Table 2. Effects of aspirin on LPS-induced pregnancy outcomes during the second trimester.

Group	Mice (n)	Dose (mg/kg)	Preterm delivery (GD < 18 days)	Term delivery (GD 18–20 days)	Preterm birth rate (%)	Live fetal mice	Dead fetal mice	Death rate (%)	P value (vs. Control)
Control	12	/	0	12	0	190	2	1.05	–
LPS	12	75 μg/kg	11	1	91.7	193	9	4.67	<0.001
Low-dose aspirin	12	0.21 mg/kg	0	12	0	220	3	1.36	1.000
High-dose aspirin	12	0.78 mg/kg	0	12	0	226	5	2.21	1.000
LPS + Low-dose aspirin	12	0.21 mg/kg	8	4	66.7	218	6	2.75	0.001
LPS+High-dose aspirin	12	0.78 mg/kg	5	7	41.7	228	7	3.07	0.012

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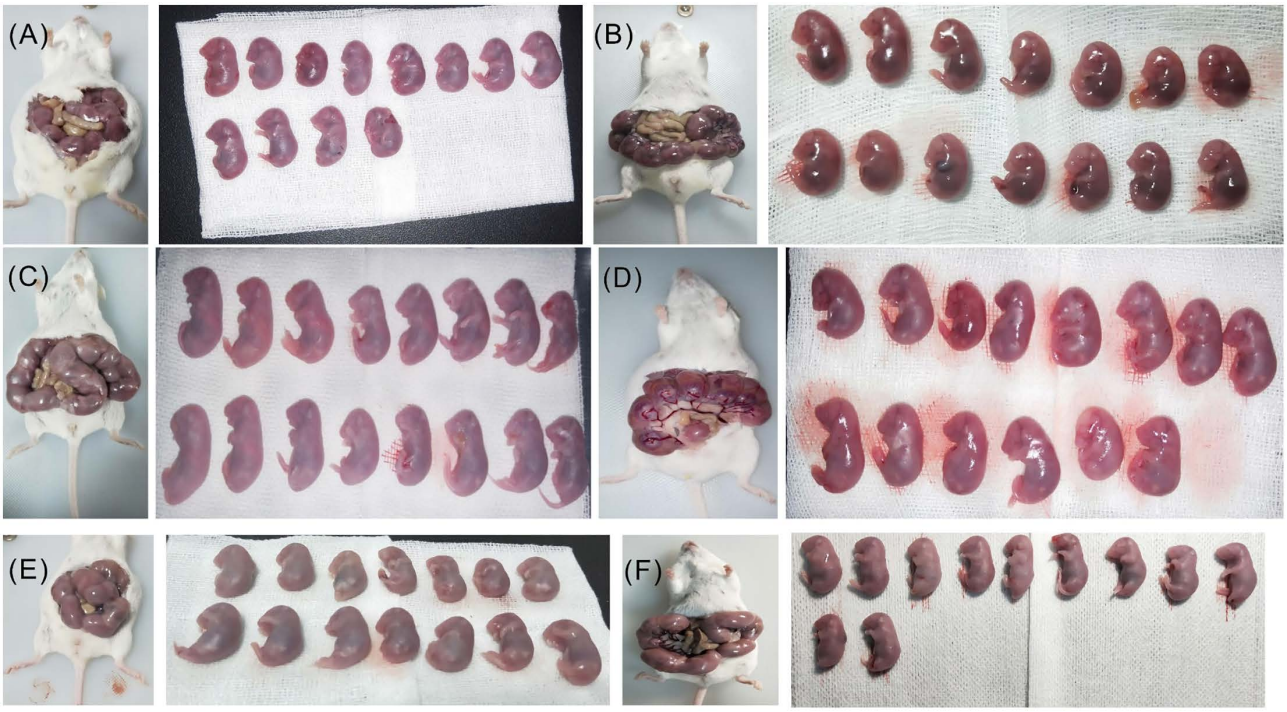

Fig 1. Picture of fetal mice appearance (On the day of delivery). (A) Pregnancy control group. (B) LPS group. (C) High-dose aspirin control group. D) Low-dose aspirin control group. (E) LPS+High-dose aspirin treatment. (F) LPS + Low-dose aspirin treatment. The neonatal mice in the LPS group often showed delayed development, a smaller body size, and abnormal skin color.

https://doi.org/10.1371/journal.pone.0344041.g001

3.2 Effects of different doses of aspirin on levels of inflammatory factors in serum, amniotic fluid and placenta of LPS preterm mice

The effects of different doses of aspirin on the elevated levels of inflammatory cytokines in serum and amniotic fluid of pregnant mice exposed to LPS in the second trimester were further studied. As shown in Fig 2A-2C and Table 3, compared with the control group, LPS exposure significantly increased serum TNF-α (8.19 ± 1.49 pg/mL vs 38.07 ± 5.35 pg/mL, P = 0.009), IL-1β (0.71 ± 0.59 pg/mL vs 37.35 ± 8.15 pg/mL), P = 0.007) and IL-6 (6.67 ± 1.51 pg/mL vs 35.04 ± 6.39 pg/mL, P = 0.021). After high-dose aspirin supplementation, LPS upregulated serum TNF-α (38.07 ± 5.35 pg/mL vs 18.59 ± 1.88 pg/mL). P = 0.007), IL-1β (37.35 ± 8.15 pg/mL vs 15.55 ± 2.16 pg/mL P = 0.013) and IL-6 (35.04 ± 6.39 pg/mL vs 12.3 ± 1.64 pg/mL P = 0.0021) were significantly inhibited. As illustrated in Fig 2A-2C and Table 3, compared with the LPS-challenged group, treatment with either TAK-242 or BAY 11−7082 significantly downregulated the serum concentrations of LPS-induced pro-inflammatory cytokines. Specifically, in the LPS + TAK-242 group, the serum levels of TNF-α, IL-1β, and IL-6 were determined to be 18.57 ± 1.98 pg/mL, 16.70 ± 2.05 pg/mL, and 14.10 ± 1.88 pg/mL, respectively, with statistical significance observed for all three cytokines when compared to the LPS group (all P < 0.05). The same trend was observed in the amniotic fluid and placenta (Table 4 and 5), where LPS-upregulated TNF-α (12.75 ± 1.07 pg/mL vs 6.8 ± 0.91 pg/mL) after high-dose aspirin supplementation. P = 0.018), IL-1β (25.51 ± 1.91 pg/mL vs 15.62 ± 1.21 pg/mL, P = 0.013) and IL-6 (84.72 ± 6.94 pg/mL vs 27.33 ± 3.29 pg/mL, P = 0.004) was significantly inhibited (Fig 2D-2F). In the placenta, with a high dose of aspirin, LPS up-regulated TNF-α (2.68 ± 0.55 vs 1.08 ± 0.16, P = 0.035), IL-1β (2.46 ± 0.26 vs 1.23 ± 0.12), P = 0.012) and IL-6 levels (2.24 ± 0.47 vs 1.07 ± 0.1 P = 0.048) were significantly inhibited (Fig 2G-2I). The expression patterns of TNF-α, IL-1β, and IL-6 in the placentas of pregnant mice analyzed by RT-qPCR were consistent with those

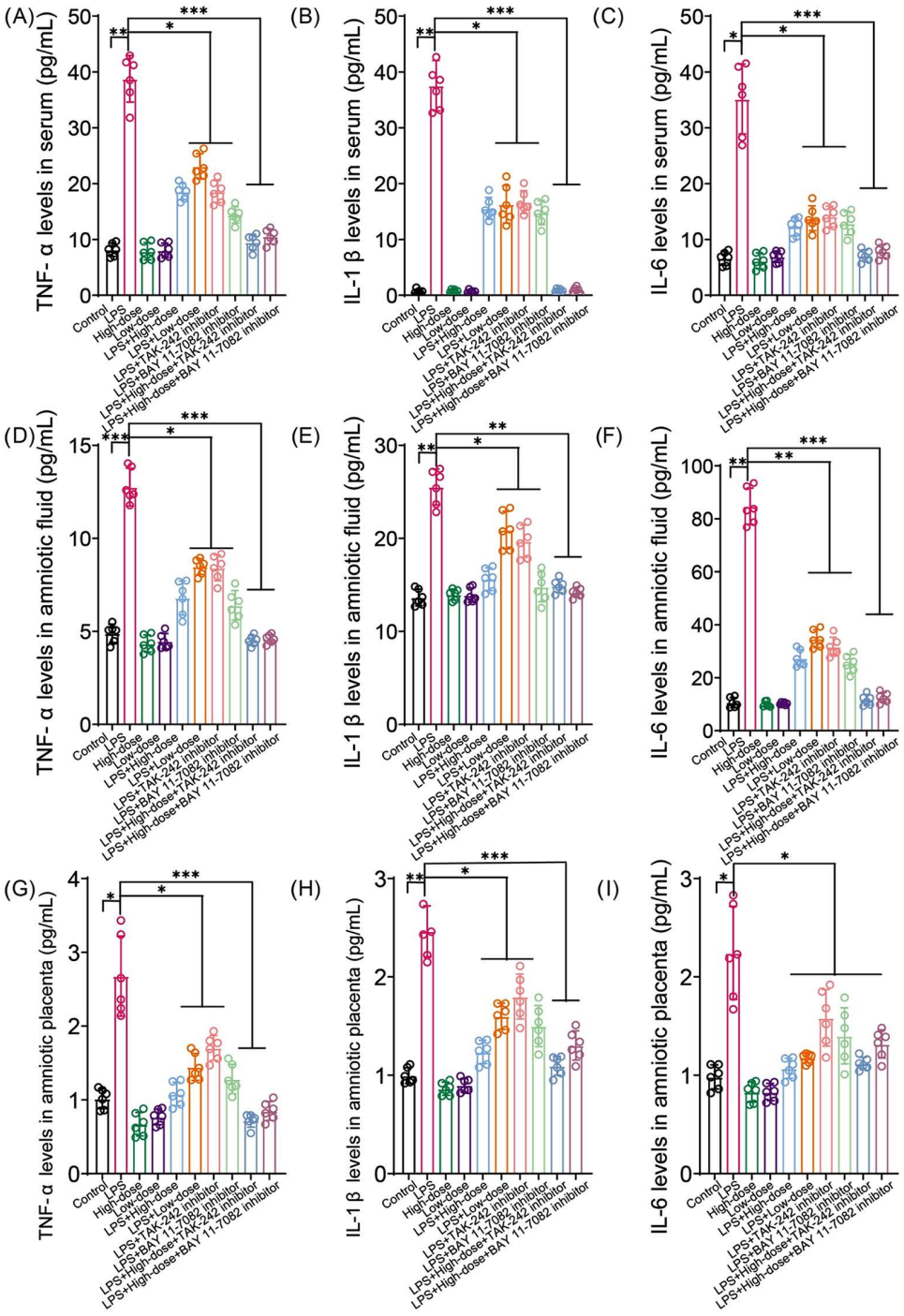

Fig 2. Effects of aspirin doses on LPS-exposed pregnant mice inflammatory cytokines. (A-C) Levels of TNF-a, IL-1β and IL-6 in serum of pregnant mice in different experimental groups. (D-F) Levels of TNF-a, IL-1β and IL-6 in amniotic fluid of pregnant mice in different experimental groups. (G-I) Levels of TNF-a, IL-1β and IL-6 in placenta of pregnant mice in different experimental groups. (Data is displayed as ± standard deviation. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001).

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Table 3. The effects of different experimental groups on the inflammatory cytokines in the serum of pregnant mice exposed to LPS.

sample/ group		Control	LPS	High-dose	Low-dose	LPS+High-dose
Serum	TNF-α	8.19 ± 1.49	38.07 ± 5.35	7.9 ± 1.54	8.04 ± 1.29	18.59 ± 1.88
	IL-1β	0.71 ± 0.59	37.35 ± 8.15	0.74 ± 0.30	0.65 ± 0.28	15.55 ± 2.16
	IL-6	6.67 ± 1.51	35.04 ± 6.39	6.17 ± 1.43	6.92 ± 1.03	12.3 ± 1.64
		LPS + Low-dose	LPS + TAK-242 inhibitor	LPS + BAY 11–7082 inhibitor	LPS + High-dose +TAK-242 inhibitor	LPS + High-dose + BAY 11–7082 inhibitor
	TNF-α	23.10 ± 2.27	18.57 ± 1.98	14.38 ± 1.45	9.5 ± 1.41	10.55 ± 1.34
	IL-1β	16.30 ± 3.37	16.7 ± 2.05	14.8 ± 2.15	0.84 ± 0.29	0.97 ± 0.47
	IL-6	13.8 ± 2.23	14.1 ± 1.88	12.9 ± 2.10	7.1 ± 1.05	7.88 ± 1.16

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Table 4. The effects of different experimental groups on inflammatory cytokines in the amniotic fluid of pregnant mice exposed to LPS.

sample/ group		Control	LPS	High-dose	Low-dose	LPS+High-dose
amniotic fluid	TNF-α	4.34 ± 1.54	12.75 ± 1.07	4.16 ± 0.66	4.40 ± 0.42	6.8 ± 0.91
	IL-1β	13.65 ± 0.89	25.51 ± 1.91	13.97 ± 0.58	13.91 ± 0.78	15.62 ± 1.21
	IL-6	10.62 ± 1.92	84.72 ± 6.94	9.92 ± 1.09	10.21 ± 0.51	27.33 ± 3.29
		LPS + Low-dose	LPS + TAK-242 inhibitor	LPS + BAY 11–7082 inhibitor	LPS + High-dose +TAK-242 inhibitor	LPS + High-dose + BAY 11–7082 inhibitor
	TNF-α	8.13 ± 1.02	8.21 ± 0.83	6.63 ± 0.94	5.16 ± 1.71	5.37 ± 2.05
	IL-1β	20.9 ± 2.01	19.7 ± 1.73	14.8 ± 1.61	14.88 ± 0.72	14.2 ± 0.54
	IL-6	34.71 ± 3.38	31.70 ± 3.57	25.3 ± 3.45	11.6 ± 2.02	12.5 ± 1.95

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Table 5. The effects of different experimental groups on inflammatory factors in the placentas of pregnant mice exposed to LPS.

sample/ group		Control	LPS	High-dose	Low-dose	LPS+High-dose
placenta	TNF-α	1.02 ± 0.14	2.68 ± 0.55	0.67 ± 0.16	0.77 ± 0.11	1.08 ± 0.16
	IL-1β	1.00 ± 0.08	2.46 ± 0.26	0.86 ± 0.07	0.9 ± 0.07	1.23 ± 0.12
	IL-6	0.98 ± 0.12	2.24 ± 0.47	0.83 ± 0.10	0.83 ± 0.09	1.07 ± 0.10
		LPS + Low-dose	LPS + TAK-242 inhibitor	LPS + BAY 11–7082 inhibitor	LPS + High-dose +TAK-242 inhibitor	LPS + High-dose + BAY 11–7082 inhibitor
	TNF-α	1.45 ± 0.18	1.70 ± 0.17	1.28 ± 0.20	0.72 ± 0.09	0.85 ± 0.13
	IL-1β	1.6 ± 0.13	1.8 ± 0.23	1.5 ± 0.21	1.10 ± 0.10	1.30 ± 0.15
	IL-6	1.18 ± 0.05	1.58 ± 0.29	1.4 ± 0.28	1.12 ± 0.06	1.32 ± 0.14

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determined by ELISA (S2 Fig in S1 File). It is worth noting that the preventive effect of aspirin is related to its dose, and in this experiment, the therapeutic effect of high-dose aspirin is better than that of low-dose aspirin.

3.3 Effects of different doses of aspirin on oxidative stress levels in liver of preterm mice with LPS

As shown in Fig 3A-B, compared with the blank control group, the serum Superoxide dismutase (SOD) level of pregnant mice in the LPS-infected group was significantly decreased (8.38 ± 0.79 ng/mL vs 2.11 ± 0.54 ng/mL, p = 0.003). Malondialdehyde (MDA) levels were significantly increased (62.48 ± 9.95 ng/mL vs 259.57 ± 34.73 ng/mL, p = 0.008). Compared with LPS group, SOD levels in blood of pregnant mice in low-dose and high-dose aspirin treatment groups were significantly increased (6.85 ± 0.51 ng/mL vs 2.11 ± 0.54 ng/mL, p = 0.005; 4.74 ± 0.68 ng/mL vs 2.11 ± 0.54 ng/mL, p = 0.007), the level

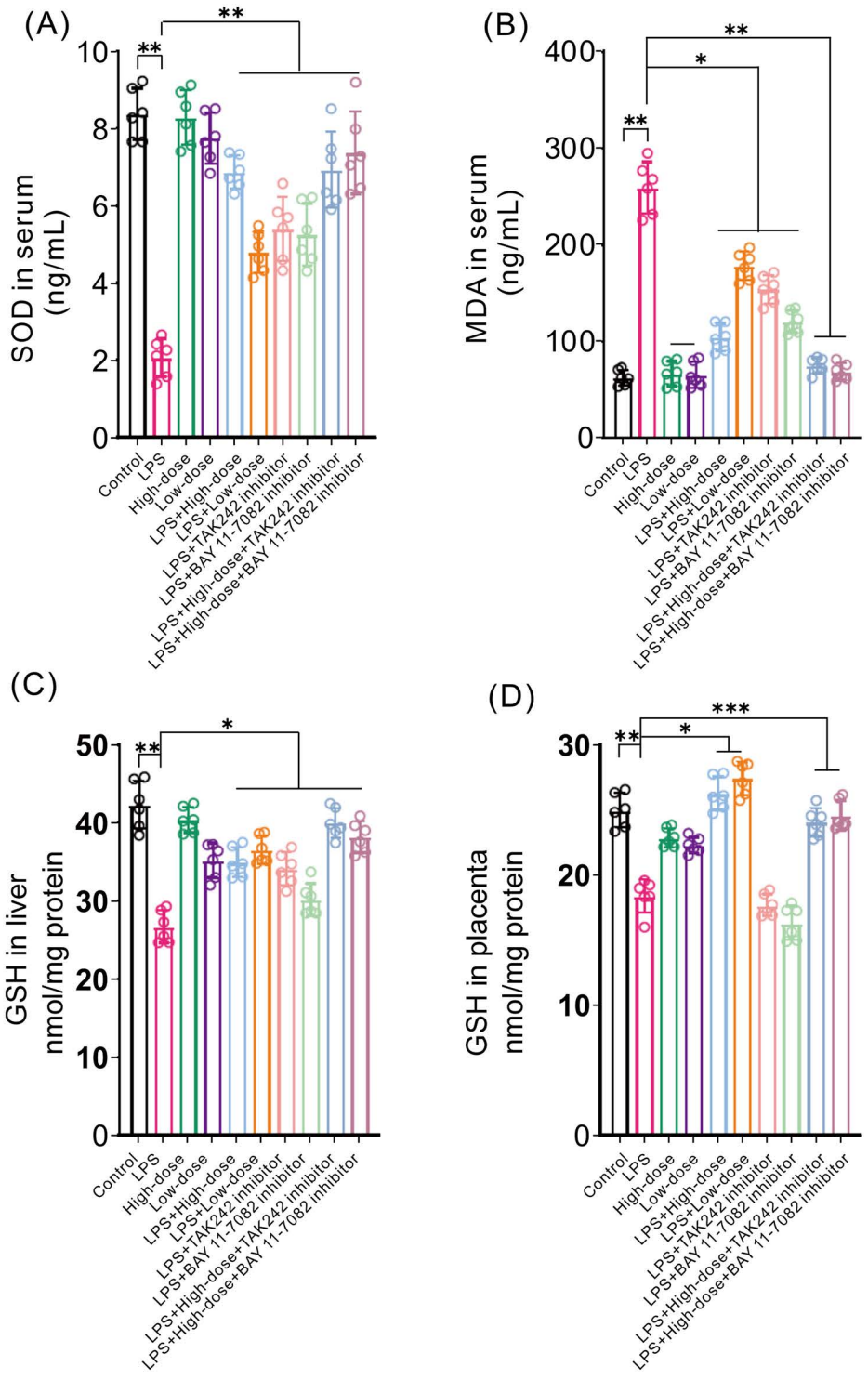

Fig 3. Effects of different doses of aspirin on oxidative stress factors in pregnant mice exposed to LPS. (A-B) Analysis of SOD and MDA content in serum. (C) Analysis of liver GSH content in pregnant mice. (D) Analysis of GSH content in placenta of pregnant mice. (Data is displayed as ± standard deviation. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001).

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of MDA was significantly decreased (185.63 ± 23.15 ng/mL vs 259.57 ± 34.73 ng/mL, p = 0.023; 104.47 ± 16.55 ng/mL vs 259.57 ± 34.73 ng/mL, p = 0.017).

This study also investigated the effects of different doses of aspirin on liver and placental GSH depletion induced by LPS exposure in pregnant mice. As shown in Fig 3C-D, compared with the control group, LPS treatment reduced the liver GSH content of pregnant mice (42.73 ± 3.02 nmol/mg vs 26.93 ± 2.1 nmol/mg, p = 0.006). Supplementation of high-dose aspirin alleviated liver GSH depletion induced by LPS (36.89 ± 1.69 nmol/mg vs 26.93 ± 2.1 nmol/mg, p = 0.023). LPS-induced placental GSH depletion was also prevented (27.42 ± 1.26 nmol/mg vs 19.06 ± 1.13 nmol/mg, p = 0.028).

3.4 Effects of aspirin on the expressions of MyD88, p-IκB, IκB, and nuclear NF-κB p65 in the placenta of LPS-induced preterm mice

As shown in Fig 4A-C and S3-S6 Fig in S1 File, compared with the control group, LPS significantly upregulated MyD88 protein level in placental tissue of pregnant mice (p = 0.009). The level of MyD88 protein in placenta could be significantly inhibited after high-dose aspirin supplementation (p = 0.013). The same trend was also confirmed at the level of p-IκB protein expression. Compared with the control group, LPS significantly upregulated p-IκB protein level in the placental tissue of pregnant mice (p = 0.004), and the placental p-IκB protein level was significantly inhibited after high-dose aspirin supplementation (p = 0.008) (Fig 4D-E).

To further elucidate the underlying mechanism, we detected the expression levels of phosphorylated IκB (p-IκB), total IκB (IκB), and nuclear NF-κB p65 protein in the following groups: Control group, LPS-treated group, LPS + High-dose aspirin group, TLR4 inhibitor (TAK-242)-treated group, NF-κB inhibitor (BAY 11–7082)-treated group, TAK-242 + High-dose aspirin combination group, and BAY 11–7082 + High-dose aspirin combination group. As shown in Fig 5 and S7-S11 Fig in S1 File, compared with the Control group, the LPS-treated group exhibited a significant increase in the relative expression level of p-IκBα, a marked decrease in total IκB, and a significant elevation in the nuclear NF-κB p65 (nuclear p65) relative expression. In the LPS + TAK-242 group (LPS combined with TLR4 inhibitor treatment) and LPS + BAY 11–7082 group (LPS combined with NF-κB inhibitor treatment), the relative expression levels of p-IκBα and nuclear p65 were significantly reduced compared with the LPS group, while the IκBα level was notably restored.

4. Discussion

This study successfully established an LPS-induced inflammatory preterm birth model in mice. The results showed that the preterm birth rate in the 75 μg/kg LPS group reached 91.7%, with a stillbirth rate of 4.67%, confirming that inflammation can significantly induce preterm birth. Aspirin, at both low and high doses, effectively reduced the preterm birth rate and increased the live birth rate ($P < 0.05$). These findings support existing clinical data, wherein low-dose aspirin has been demonstrated to reduce the risk of preterm birth, particularly in high-risk populations [34,39–42,51]. For instance, a randomized trial showed that low-dose aspirin reduced the risk of spontaneous preterm birth before 34 weeks by 57% (OR 0.43, 95% CI 0.26–0.84) [34], and another study also reported that aspirin was associated with a reduction in spontaneous preterm birth (marginal relative risk 0.70, 95% CI 0.57–0.86) [41]. Mechanistically, aspirin treatment reversed the LPS-induced decline in GSH levels and inhibited NF-κB activation, as well as the expression of downstream inflammatory factors such as TNF-α, IL-6, and IL-1β [33]. This suggests that aspirin exerts its protective effects by modulating oxidative stress and inflammatory signaling pathways. This aligns with the known anti-inflammatory properties of aspirin in the literature, such as its ability to reduce inflammatory events by inhibiting prostaglandin synthesis and COX enzyme activity [52].

Notably, inflammatory stimulation (LPS) was found to induce skeletal developmental abnormalities in fetal mice, including occipital hypoplasia, costosternal malformations, and bone absence—effects that were alleviated by aspirin exposure. While no direct evidence in the existing literature addresses aspirin's role in offspring skeletal development, its well-documented anti-inflammatory properties may indirectly mitigate such developmental anomalies. Specifically, aspirin could

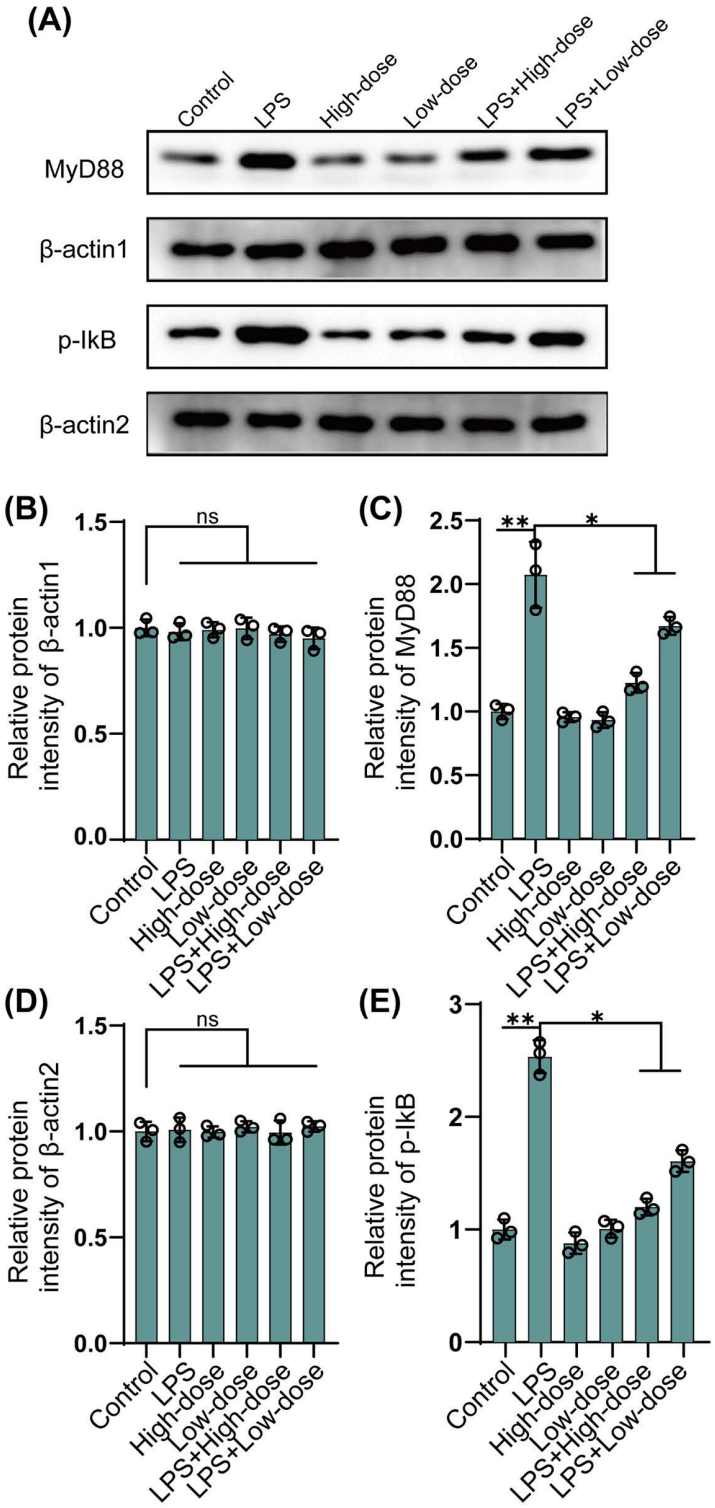

Fig 4. Representative Western blot images and quantitative analysis of MyD88 and p-IκB in placenta. (A) Western blot analysis of MyD88 and p-IκB protein expression in placental tissue of the following experimental group: Control, LPS, High-dose control group, Low-dose control group, LPS+High-dose, LPS + Low-dose. (B-E) Statistical analysis of β-actin1, MyD88, β-actin2 and p-IκB intensity in (A) by ImageJ software. (Data is displayed as ± standard deviation. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001).

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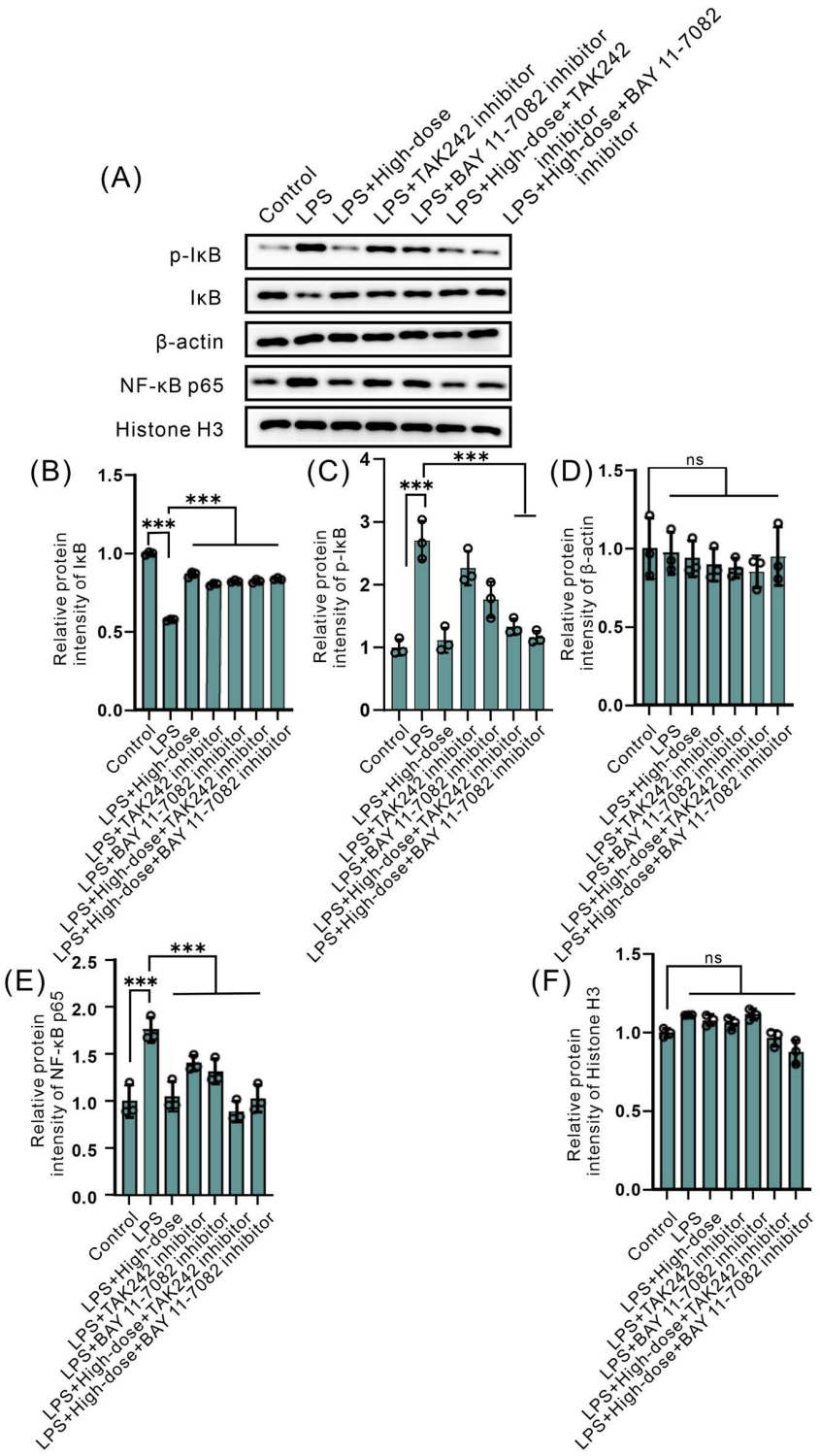

Fig 5. Representative Western blot images and quantitative analysis of p-IκB, IκB, and nuclear NF-κB p65. (A) Western blot analysis of p-IκB, IκB, β-actin, nuclear NF-κB p65 and Histone H3 in seven groups: Control, LPS, High-dose control group, Low-dose control group, LPS+High-dose, LPS + Low-dose. (B-F) Statistical analysis of p-IκB, IκB, β-actin, nuclear NF-κB p65 and Histone H3 by ImageJ software. (Data is displayed as ± standard deviation. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001).

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downregulate proinflammatory mediators (e.g., TNF-α, IL-6), thereby potentially modulating fetal tissue differentiation through the maternal-fetal interface [53,54]. However, the specific mechanisms, such as whether it directly affects bone cell differentiation and metabolism, require further exploration, highlighting the significance of this study in expanding the scope of aspirin's effects.

At the molecular level, aspirin significantly reversed the LPS-induced upregulation of MyD88 and p-IκB expression and reduced p65 nuclear translocation, suggesting that aspirin may exert its anti-inflammatory effects by interfering with the TLR4/NF-κB pathway [22,23]. Administration of a TLR4 inhibitor downregulated the serum levels of proinflammatory cytokines (e.g., TNF-α and IL-6) as well as the protein expressions of Nuclear NF-κB p65 and p-IκB, further confirming that the TLR4 pathway is a key target mediating the effects of aspirin. Treatment with an NF-κB inhibitor significantly reduced the protein levels of NF-κB p65 (including its nuclear translocation) and phosphorylated IκB (p-IκB), which directly validates that the NF-κB pathway serves as a critical mediator of inflammatory responses in our LPS-induced preterm mouse model. These findings are consistent with aspirin's immunomodulatory properties reported in the literature, such as its role in reducing inflammation via similar pathways in preterm birth prevention [55]. However, this study provides more in-depth evidence from an animal model, addressing the lack of mechanistic validation in clinical research.

The significance of this study lies in several aspects: First, it directly validates through an animal model that aspirin reduces preterm birth via the TLR4/NF-κB pathway, whereas existing literature mostly offers indirect inferences. Second, this study is the first to report aspirin's protective effects against fetal skeletal developmental abnormalities, expanding its potential applications in obstetrics. Existing literature primarily focuses on preterm birth prevention rather than developmental impacts. Finally, the findings support the value of aspirin as an anti-inflammatory agent in preventing preterm birth, particularly providing experimental basis for high-risk populations (e.g., women with a history of preterm birth) [40,56]. However, emerging evidence indicates that the efficacy of aspirin may be influenced by factors such as genetic polymorphisms, concurrent medications, individual disease status, smoking habits, and dietary patterns [57,58]. Therefore, future studies need to optimize dosage and timing and advance to human trials for validation. In summary, this study not only reinforces the anti-inflammatory mechanisms of aspirin but also provides an important theoretical foundation for developing new strategies for preterm birth prevention.

It is important to acknowledge certain limitations of the present study. Firstly, the experiments were exclusively conducted in a mouse model. Notably, the LPS-induced preterm birth model has inherent limitations in recapitulating the complex etiology of human preterm birth, which often involves multiple pathogens or damage-associated molecular patterns (DAMPs) [59,60]. Given the well-documented physiological and pathological discrepancies between mice and humans, the translatability of our findings to clinical settings may be constrained, as the failure rate for translating drug effects from animal models to human therapies remains over 92%. Secondly, this study only assessed the therapeutic efficacy of aspirin at a single dose and administration time point; the influence of varying doses and timing regimens-factors critical to optimizing clinical utility-has not been comprehensively elucidated. To address these limitations, future research should include multi-species animal models (e.g., non-human primates) to further validate the efficacy and safety of aspirin for preterm birth prevention. Additionally, systematic investigations into dose-response relationships and optimal administration windows are warranted to provide more robust evidence for clinical translation.

5. Conclusions

This study confirmed that aspirin reduces inflammatory factors, ameliorates pathological damage, and inhibits NF-κB phosphorylation and nuclear translocation, thereby preventing inflammatory preterm birth through suppression of the TLR4–NF-κB pathway. These findings provide a theoretical basis for the application of aspirin in the prevention and treatment of preterm birth. Therefore, future research should focus on the dosage and exposure time of aspirin, validation of mechanisms in clinical samples, the impact of inflammatory factors on fetal development, and the protective mechanisms of aspirin.

Supporting information

S1 File. S1 Fig. Skeletal diagram of fetal mouse. A) Skeletons of fetuses from the control group. B) LPS-induced deformities of the skeleton in mice. C) Skeletons of fetuses from high-dose aspirin treatment group. D) Skeletons of fetuses from low-dose aspirin treatment group. **S2 Fig.** qPCR was used to detect the relative mRNA expression levels of key inflammatory factors in placental tissues from different groups. GAPDH was served as the internal reference gene, and the relative expression levels of target genes were calculated by the $2^{-}\Delta\Delta Ct$ method. (A) Relative mRNA expression level of Tumor Necrosis Factor-α (TNF-α); (B) Relative mRNA expression level of Interleukin-1β (IL-1β); (C) Relative mRNA expression level of Interleukin-6 (IL-6). Differences between groups were compared using an t-test. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate statistically significant differences between groups. **S3 Fig.** The original blot and gel (MyD88). **S4 Fig.** The original blot and gel (β-actin-1). **S5 Fig.** The original blot and gel (p-IκB). **S6 Fig**. The original blot and gel (β-actin-2). **S7 Fig.** The original blot and gel (p-IκB). **S8 Fig.** The original blot and gel (IκB). **S9 Fig.** The original blot and gel (β-actin). **S10 Fig.** The original blot and gel (nuclear NF-κB p65). **S11 Fig**. The original blot and gel (Histone H3).
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