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Betamethasone as a potential treatment for preterm birth associated with sterile intra-amniotic inflammation: a murine study

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Abstract

Introduction: Preterm birth remains the leading cause of perinatal morbidity and mortality worldwide. Preterm birth is preceded by spontaneous preterm labor, which is commonly associated with sterile intra-amniotic inflammation; yet, no approved treatment exists for this clinical condition. Corticosteroids are the standard of care to improve neonatal outcomes in women at risk of preterm birth. Herein, we first validated our model of alarmin-induced preterm birth. Next, we investigated whether treatment with betamethasone could prevent preterm birth resulting from sterile intra-amniotic inflammation in mice.

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Methods: Under ultrasound guidance, the first cohort of dams received an intra-amniotic injection of the alarmin high-mobility group box-1 (HMGB1, n=10) or phosphate-buffered saline (PBS, n=9) as controls. Next, a second cohort of dams received HMGB1 intra-amniotically and were subcutaneously treated with betamethasone (n=15) or vehicle (n=15). Dams were observed until delivery, and perinatal outcomes were observed.

Results: Intra-amniotic HMGB1 reduced gestational length (p=0.04), inducing preterm birth in 40% (4/10) of cases, of which 100% (4/4) were categorized as late preterm births. Importantly, treatment with betamethasone extended the gestational length (p=0.02), thereby reducing preterm birth by 26.6% [from 33.3% (5/15) to 6.7% (1/15)]. Treatment with betamethasone did not worsen the rate of neonatal mortality induced by HMGB1 or alter weight gain in the first three weeks of life.

Conclusion: Treatment with betamethasone prevents preterm birth induced by the alarmin HMGB1. This study supports the potential utility of betamethasone for treating women with sterile intra-amniotic inflammation.

Keywords

Alarmins; Antenatal corticosteroids; HMGB1; Preterm labor; Prematurity; Pregnancy

INTRODUCTION

Preterm birth remains one of the foremost causes of perinatal morbidity and mortality globally [1–4]. Preterm birth occurs after spontaneous preterm labor in approximately 70% of cases [5]. While several etiologies have been described for the syndrome of spontaneous preterm labor [6], the only well-established causal link is intra-amniotic infection and/or inflammation [7–21]. While intra-amniotic infection is caused by microbes invading the amniotic cavity [8, 9, 11, 22–37], it is now well-accepted that sterile intra-amniotic inflammation is triggered by non-microbial stimuli [35, 38–46].

Sterile inflammation is driven by damage-associated molecular patterns (DAMPs) or alarmins [47–51], which include high-mobility group box-1 (HMGB1) [52, 53], interleukin (IL)-1α [54], heat-shock protein 70 (HSP70) [55], and S100 calcium-binding protein (S100B) [56]. Importantly, sterile intra-amniotic inflammation is commonly diagnosed in women who underwent spontaneous preterm labor with intact membranes [40]. Moreover, women who had high concentrations of HMGB1 in amniotic fluid and sterile intra-amniotic inflammation underwent earlier delivery than women with low concentrations of this alarmin [40]. In tandem with clinical observations, murine studies demonstrated that the intra-amniotic injection of HMGB1, S100B, or IL-1α causes preterm delivery [15, 21, 57], thereby establishing a causal link between intra-amniotic inflammation triggered by alarmins and spontaneous preterm birth. However, to date there are no approved treatment regimens for patients with sterile intra-amniotic inflammation.

The administration of antenatal corticosteroids such as betamethasone has become the standard of care for women at risk of delivering preterm within one week, regardless of the underlying cause [58]. Corticosteroids are recommended as they have been shown to accelerate fetal organ maturation, not only diminishing the rate of Respiratory Distress Syndrome and intraventricular hemorrhage but, more importantly, reducing the risk of perinatal and neonatal death [59]. Notably, pre-treatment with betamethasone has also been shown to delay delivery and reduce amniotic fluid cytokine concentrations using an *in vivo* model of preterm birth caused by systemic endotoxin administration [60]. However, the effects of betamethasone in the clinical setting of sterile intra-amniotic inflammation have not been evaluated.

In the current study, we first validated our established *in vivo* model of sterile intra-amniotic inflammation induced by the administration of the alarmin HMGB1 under ultrasound guidance. Using this model, we examined the potential utility of betamethasone treatment for preventing preterm birth.

METHODS

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in the vivarium of our institution. All mice were maintained under a circadian cycle (light:dark = 12:12 h). Females (8-12 weeks old) were mated with males of demonstrated fertility. Female mice were checked every morning between 8:00 - 9:00 a.m. for the appearance of a vaginal plug, which designated 0.5 days *post coitum* (dpc). Dams were then housed separately from the males and their weights were checked daily. An increase in weight of ≥2 grams by 12.5 dpc confirmed pregnancy. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) (Protocol No. 18-03-0584).

Intra-amniotic administration of HMGB1

The intra-amniotic administration of HMGB1 was performed as previously described [57]. Briefly, dams were anesthetized on 14.5 dpc by inhalation of 2% isoflurane [Fluriso™ (Isoflurane, USP) Vetone Boise, ID, USA] and 1–2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.5–2% isoflurane and 1.5–2 L/min of oxygen. Mice were positioned on a heating pad and stabilized with adhesive tape. Fur was removed from the abdomen and thorax by applying Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA). Body temperature was maintained at 37 ± 1°C as indicated by rectal thermometer (VisualSonics, Toronto, ON, Canada), and respiratory and heart rates were monitored by electrodes embedded in the heating pad. The ultrasound transducer was placed in a mechanical holder, and was slowly moved towards the abdomen. Ultrasound-guided intra-amniotic injection was performed in each amniotic sac using recombinant human HMGB1 (Biolegend, San Diego, CA, USA) dissolved in sterile 1X phosphate-buffered saline (PBS; Life Technologies, Grand Island, NY, USA) at a concentration of 9 ng/100 μL using a 30-G needle (BD PrecisionGlide Needle; Becton Dickinson, Franklin Lakes, NJ, USA) (n = 10). Controls were injected with 100 μL of PBS alone (n = 9). Color Doppler ultrasound was used to identify the "injection jet sign" [61], which confirmed that the fluid was injected inside of the amniotic cavity (Figure 1A). Following the ultrasound, dams were positioned under a heat lamp for recovery. Afterwards, dams were monitored *via* video camera until delivery to evaluate pregnancy outcomes.

Betamethasone treatment of mice intra-amniotically injected with HMGB1

Dams received an intra-amniotic injection of HMGB1 in each amniotic sac on 14.5 dpc under ultrasound guidance. Dams were then treated subcutaneously with either 0.1 mg/100 μL of Betamethasone Sodium Phosphate and Betamethasone Acetate Injectable Suspension (American Regent Inc. Shirley, NY, USA; n = 15) diluted in vehicle control [0.9% sodium chloride injection (saline), Hospira, Lake Forest, IL, USA] or vehicle control alone (n = 15) at 12 hours (h) and 36 h after the intra-amniotic injection of HMGB1. Dams were then observed *via* video camera until delivery to evaluate pregnancy and neonatal outcomes. The betamethasone dosage was chosen based on prior reports indicating that 0.1 mg of betamethasone induced lung maturation in mice and was equivalent to the two 12 mg doses used in pregnant women [60, 62]. The latter dose and schedule is used as the standard of care in pregnant women who are predicted to be at risk of preterm delivery within one week [58].

Video monitoring and pregnancy and neonatal outcomes

Pregnancy parameters, which included the preterm birth rate and neonatal mortality rate were recorded using a video camera (Sony Corporation, Tokyo, Japan). The gestational length was determined as the time period from the observation of the vaginal plug until the delivery of the first pup. Preterm birth was defined as delivery <19.0 dpc, and this rate was calculated as the percentage of dams delivering <19.0 dpc among the total number of injected dams. Late preterm birth was defined as delivery between 18.0 - 19.0 dpc, and early preterm birth as delivery <18.0 dpc (Figure 1A). The neonatal weights were reported as the mean weight of all neonates from a single dam (litter). The neonatal mortality rate for each litter was calculated using the number of dead delivered pups among the total litter size. Neonates were observed until three weeks postpartum to evaluate neonatal weight and survival.

Statistical analysis

GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com) was used to conduct statistical analysis. The Fisher's exact test was used for the rates of preterm birth, the Mann–Whitney *U*-test was used for gestational length, neonatal mortality, and neonatal weight, and Kaplan–Meier survival curves were used to plot and compare neonatal survival (Mantel–Cox test). A *p*-value <0.05 was considered statistically significant.

RESULTS

The intra-amniotic injection of HMGB1 causes preterm birth

HMGB1 is one of the most studied alarmins in the amniotic cavity [38, 40, 42, 57, 63–67] and is elevated in amniotic fluid of women with sterile intra-amniotic inflammation [40, 42]. Additionally, we previously demonstrated causality between the intra-amniotic injection of HMGB1 and preterm labor/birth [57]. Given that the bioactivity of HMGB1 is variable [68], our first aim was to validate our previous model of preterm labor/birth caused by HMGB1. Under ultrasound guidance, dams were intra-amniotically injected with

9 ng/100 µL of HMGB1 or 100 µL of PBS on 14.5 dpc and observed until delivery (Figure 1A). This dose of HMGB1 was determined from the pathophysiological amniotic fluid concentrations found in patients with sterile intra-amniotic inflammation [40]. Color Doppler ultrasound was used to identify the "injection jet sign" and confirm that the fluid was injected inside of the amniotic cavity (Figure 1A) [61]. We found that the gestational length of the dams injected with HMGB1 was decreased compared to the gestational length of controls [HMGB1: 19.16 (18.89-19.23) vs. PBS: 19.26 (19.14-19.29) dpc, p = 0.04] (Figure 1B). Specifically, 40% (4/10) of the mice injected with HMGB1 underwent preterm birth (<19.0 dpc), and all (10/10) of the control dams underwent term delivery (≥19.0 dpc) (p = 0.05) (Figure 1C). Notably, all of these preterm births were late preterm (Figure 1B), which is the period when most human preterm births occur [1, 3, 5]. Beyond pregnancy complications, we evaluated the impact of intra-amniotic HMGB1 on the neonates. No differences were found in the mortality at birth between the neonates born to dams that received an intra-amniotic injection of HMGB1 and controls [HMGB1: 37.5% (17.08-41.52) vs. PBS: 25% (14.29-27.5), p = 0.2], which is consistent with our prior study [57]. Thus, the intra-amniotic injection of HMGB1 may result in late preterm birth.

Treatment with betamethasone prevents preterm birth induced by HMGB1

Next, we investigated whether betamethasone, a corticosteroid used as the standard of care in pregnant women at risk of preterm delivery [58], could prevent preterm birth and adverse neonatal outcomes induced by HMGB1. Dams were intra-amniotically injected with HMGB1 on 14.5 dpc and subcutaneously treated with betamethasone 12 h and 36 h after intra-amniotic injection (Figure 2A). Controls were subcutaneously treated with saline. We found that the gestational length of the dams that received HMGB1 was increased after betamethasone treatment compared to the gestational length of dams that received HMGB1 and were treated with the vehicle alone [HMGB1 + Betamethasone: 19.3 (19.17-19.35) vs. HMGB1 + Saline: 19.13 (18.79-19.25) dpc, p = 0.02] (Figure 2B). Specifically, the rate of preterm birth was reduced by 26.6% [from 33.3% (5/15) to 6.7% (1/15), p = 0.08] following betamethasone treatment (Figure 2C). To our knowledge, this is the first demonstration that betamethasone can reduce the rate of preterm birth in the setting of sterile intra-amniotic inflammation.

The use of betamethasone in women with high risk of preterm birth has been shown to improve perinatal and neonatal mortality, regardless of the underlying etiology [58]. However, the specific effects of this medication in the subset of women with preterm labor and sterile-intra-amniotic inflammation is unknown. Thus, we next assessed the outcomes of neonates from dams that received HMGB1 and were treated with betamethasone compared to controls. No differences were found in the neonatal mortality rates at week one, two, and three of life (p = 0.6) (Figure 3A). Furthermore, we did not find differences in neonatal weights at week one (p = 0.2), two (p = 0.1), or three (p = 0.4) of life (Figures 3B–D). These results indicate that treatment with betamethasone does not worsen the rate of neonatal mortality induced by HMGB1 or alter the neonatal weight gain.

DISCUSSION

Principal findings of the study

In the current study, we established that the intra-amniotic administration of HMGB1 causes late preterm birth. Importantly, treatment with betamethasone, a commonly used corticosteroid in women with high risk of preterm birth, extended gestational length, preventing preterm birth. This corticosteroid neither worsened the neonatal mortality induced by HMGB1 nor altered neonatal weight gain in the first three weeks of life. These findings show the potential utility of betamethasone for the treatment of preterm birth associated with sterile intra-amniotic inflammation.

The alarmin HMGB1 drives sterile intra-amniotic inflammation

HMGB1 is a nuclear protein initially described as an important regulator of transcription and gene expression [52]. Yet, HMGB1 has also been recognized as an alarmin that is secreted by leukocytes in response to inflammatory stimuli [69–71]. HMGB1 represents a prototypical alarmin, given that it meets the proposed criteria for such molecules [49]. Growing evidence has suggested a role for HMGB1 in the induction of sterile intra-amniotic inflammation, which includes the following: 1) the concentrations of HMGB1 were significantly higher in amniotic fluid of patients with sterile intra-amniotic inflammation than in those without [38, 40]; 2) intra-amniotic HMGB1 shortens gestational length in mice, leading to preterm birth [57]; and 3) HMGB1 causes inflammasome-mediated inflammation in the chorioamniotic membranes [66, 72]. Together with the current study, these findings firmly establish HMGB1 as an alarmin that promotes sterile inflammatory responses and preterm birth.

Can sterile intra-amniotic inflammation be treated?

The treatment of sterile inflammation has been challenging. Standard methods for treating sterile inflammatory processes (e.g. gout, rheumatoid arthritis, etc.) include anti-inflammatory drugs such as non-steroidal anti-inflammatory [73] or corticosteroid [74–76] medications. Moreover, treatments that specifically decrease the concentration of danger signals driving sterile inflammation have also been utilized, such as allopurinol, a drug that decreases uric acid in patients with gout [77–79]. However, the treatment of sterile intra-amniotic inflammation is even more challenging, since the majority of drugs used to treat sterile inflammation-related diseases are not approved for use in pregnant women. Specific treatment options have been explored in mice, such as the inhibition of the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome [15, 21] This pathway is associated with the pathogenesis of sterile intra-amniotic inflammation [15, 21, 80, 81] Specifically, the blockade of the NLRP3 inflammasome was effective in diminishing the rates of preterm birth and neonatal mortality in mice [15]; however, this potential therapy has not been approved for clinical use during pregnancy [15]. Therefore, there is a growing need for drugs that are already approved for use in pregnant women and can be used to effectively treat sterile intra-amniotic inflammation. A pioneer study using catheterized rhesus macaques demonstrated that the uterine contractions caused by the intra-amniotic administration of IL-1β were reduced in macaques treated with indomethacin [82], a tocolytic agent that can also be used to delay preterm labor. Yet, indomethacin is only recommended for use until 32 weeks of gestation due to the risk of ductus arteriosus closure, limiting its utility [83–85]. Subsequently, these investigators showed that treatment with either dexamethasone or IL-10 reduced the uterine activity caused by the intra-amniotic administration of IL-1β in pregnant macaques [86]. Furthermore, recent studies have proposed the use of clarithromycin, an approved antibiotic for use in pregnancy, as part of a potential treatment regimen for intra-amniotic infection/inflammation [87–90]. Indeed, clarithromycin was also shown to be effective for decreasing the severity of intra-amniotic inflammation in patients with sterile intra-amniotic inflammation [90] and improving perinatal outcomes in an animal model of intra-amniotic infection [19]. However, further mechanistic experimentation to evaluate the effectiveness of clarithromycin in the setting of sterile intra-amniotic inflammation is required.

Betamethasone as a treatment for sterile intra-amniotic inflammation

Herein, we demonstrated for the first time that betamethasone restores the normal timing of delivery in a model of HMGB1-induced sterile intra-amniotic inflammation. Corticosteroids, both endogenous and pharmacological, exert their classic anti-inflammatory functions by traversing the plasma membrane of immune cells and binding to cytosolic corticosteroids receptors, which then regulate gene transcription and signal transduction within the inflamed target tissues [91, 92]. Interestingly, corticosteroids can also inhibit the expression and release of alarmins, including HMGB1, under various inflammatory conditions [93–98].

Corticosteroids have several different mechanisms of action for the regulation of inflammation, most of which are dependent on the timing of exposure [76]. Prior *in vivo* studies have shown that treatment with betamethasone did not decrease the inflammatory response taking place in the amniotic fluid [99], chorioamniotic membranes [99], fetal thymus [100], and fetal lungs [101, 102] in sheep with intra-amniotic inflammation induced by endotoxin. Yet, such inflammatory response was diminished when betamethasone was administered prior to the endotoxin stimuli [99–101]. However, in the clinical setting, the administration of corticosteroids before or at the onset of intra-amniotic inflammation is very unlikely. Therefore, our experimental model resembles the clinical scenario and management of sterile intra-amniotic inflammation, and the fact that betamethasone restored the timing of delivery supports its clinical administration in patients with this condition. Furthermore, one possible mechanism by which betamethasone exerts its effects on sterile intra-amniotic inflammation is by dampening PRR signaling pathways [92], thus suppressing production of the inflammatory mediators typically found in high concentrations in amniotic fluid of women with this clinical condition [38, 42, 46, 63]. Additionally, corticosteroids have been shown to attenuate leukocyte migration to damaged tissue sites by inhibiting transcription of adhesion molecules such as SELE (E-selectin), intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 [103, 104], all of which are involved in neutrophil migration to the reproductive tissues [105–107]. We have previously shown that spontaneous term labor is associated with upregulation of VCAM-1 and ICAM-1 in the choriodecidua [108], suggesting that betamethasone could delay the process of labor by preventing leukocyte migration to these tissues [109, 110]. Lastly, corticosteroids have been shown to mediate the polarization of monocytes and macrophages towards an anti-inflammatory phenotype, promoting the phagocytosis of apoptotic cells and debris [111–113]. The latter finding suggests that treatment with betamethasone could prevent aberrant pro-inflammatory (M1) polarization of macrophages at the maternal-fetal interface, which is consistent with prior reports showing that an increased fraction of M1-like macrophages in this compartment is correlated with preterm labor and birth [114]. In this regard, betamethasone may exert its beneficial effects by promoting M2 polarization of monocytes and macrophages. Furthermore, it is most likely that betamethasone has a complex role in regulation of the inflammatory response, and mitigates the consequences of sterile intra-amniotic inflammation through multiple non-exclusive mechanisms. Future studies are warranted to elucidate the mechanisms whereby betamethasone reduces sterile intra-amniotic inflammation and prevents preterm labor and birth.

In our model, treatment with betamethasone did not worsen the rate of neonatal mortality induced by HMGB1 or alter the neonatal weight gain in the first three weeks of life. This finding supports the cautious use of corticosteroids for the prevention of preterm birth. Yet, contrary to what is reported in humans [59, 115], treatment with betamethasone did not improve neonatal survival, which highlights the importance of intensive neonatal care.

It is worth mentioning that a limitation of our model is that a subset of neonates from dams that received an intra-amniotic injection, even with saline, failed to thrive. This is likely due to the invasive nature of an intra-amniotic injection on 14.5 days *post coitum*, which may have a greater impact on fetal development than that performed on 16.5 days *post coitum* [13, 15, 16, 19, 21, 116]. Yet, the intra-amniotic administration of alarmins guided by ultrasound allows us to mechanistically study the clinical syndrome of sterile intra-amniotic inflammation.

Conclusion

In this study, we provide evidence that betamethasone, a common medication approved for use during high-risk pregnancies, prevents preterm birth caused by the intra-amniotic injection of the alarmin HMGB1. These findings support the potential utility of this corticosteroid for preventing preterm birth in patients with sterile intra-amniotic inflammation.

List of Abbreviations:

DAMPs	damage-associated molecular patterns
dpc	days *post coitum*
HMGB1	high-mobility group box-1
HSP70	heat-shock protein 70
ICAM	intercellular adhesion molecule
IL	interleukin
NLRP3	NOD-like receptor family, pyrin domain containing 3

PBS	phosphate-buffered saline
PRR	pattern recognition receptors
SELE	E-selectin
S100B	S100 calcium-binding protein
VCAM	vascular cell adhesion molecule

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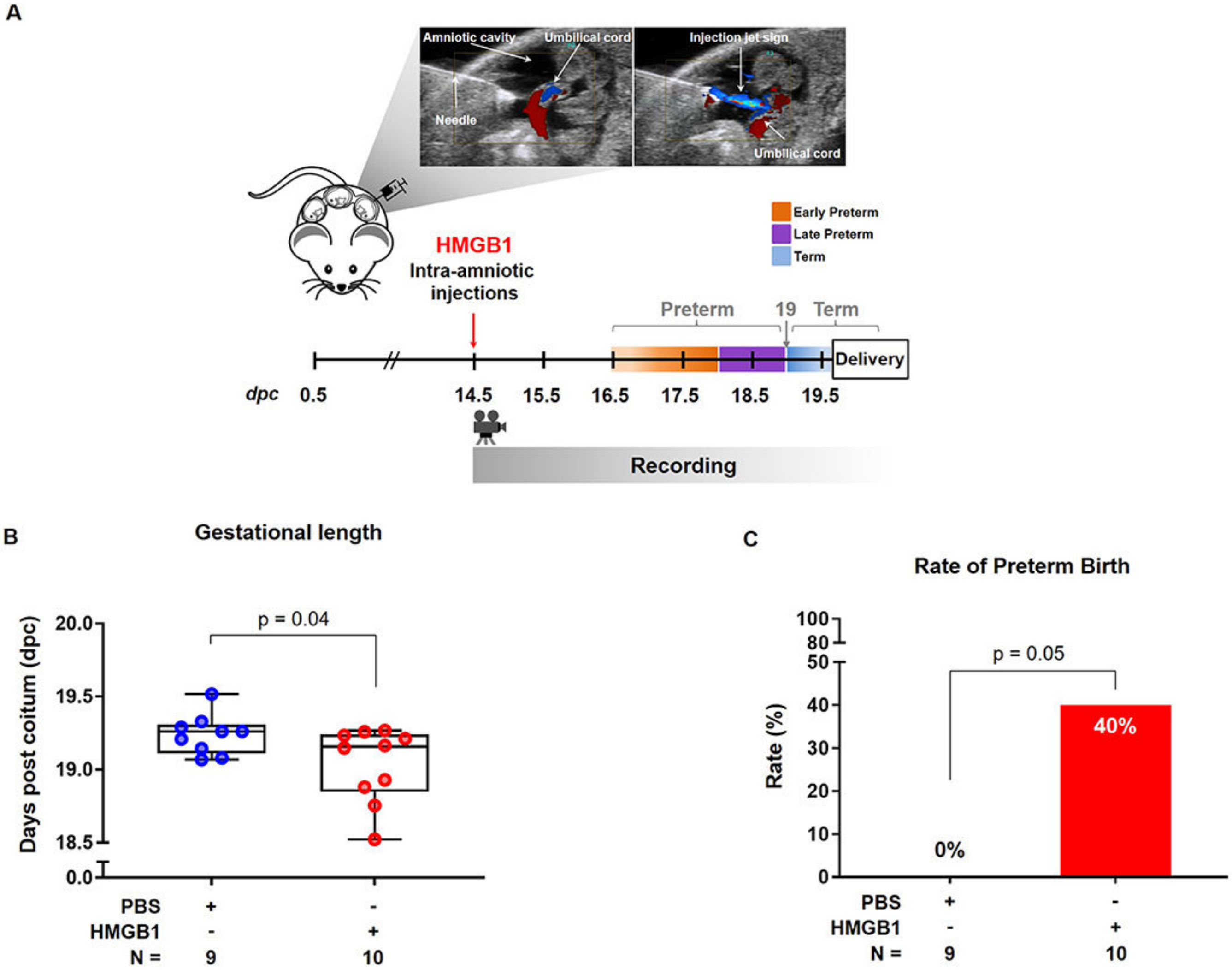

Figure 1. Intra-amniotic injection of HMGB1 guided by ultrasound.

(A) Experimental design: Dams were intra-amniotically injected with HMGB1 (9 ng/100 μL) or PBS (100 μL) on 14.5 days *post coitum* (dpc) and observed until birth. Color Doppler was used to identify the "injection jet sign." **(B)** Gestational length of dams injected with vehicle (blue dots) or HMGB1 (red dots) (n = 9-10 each). Data are shown as boxplots where the midline represents the median, boxes represent interquartile range, and whiskers represent the minimum/maximum range. **(C)** Preterm birth rate (<19 dpc) for dams that received vehicle (blue dots) or HMGB1 (red dots) (n = 9-10 each).

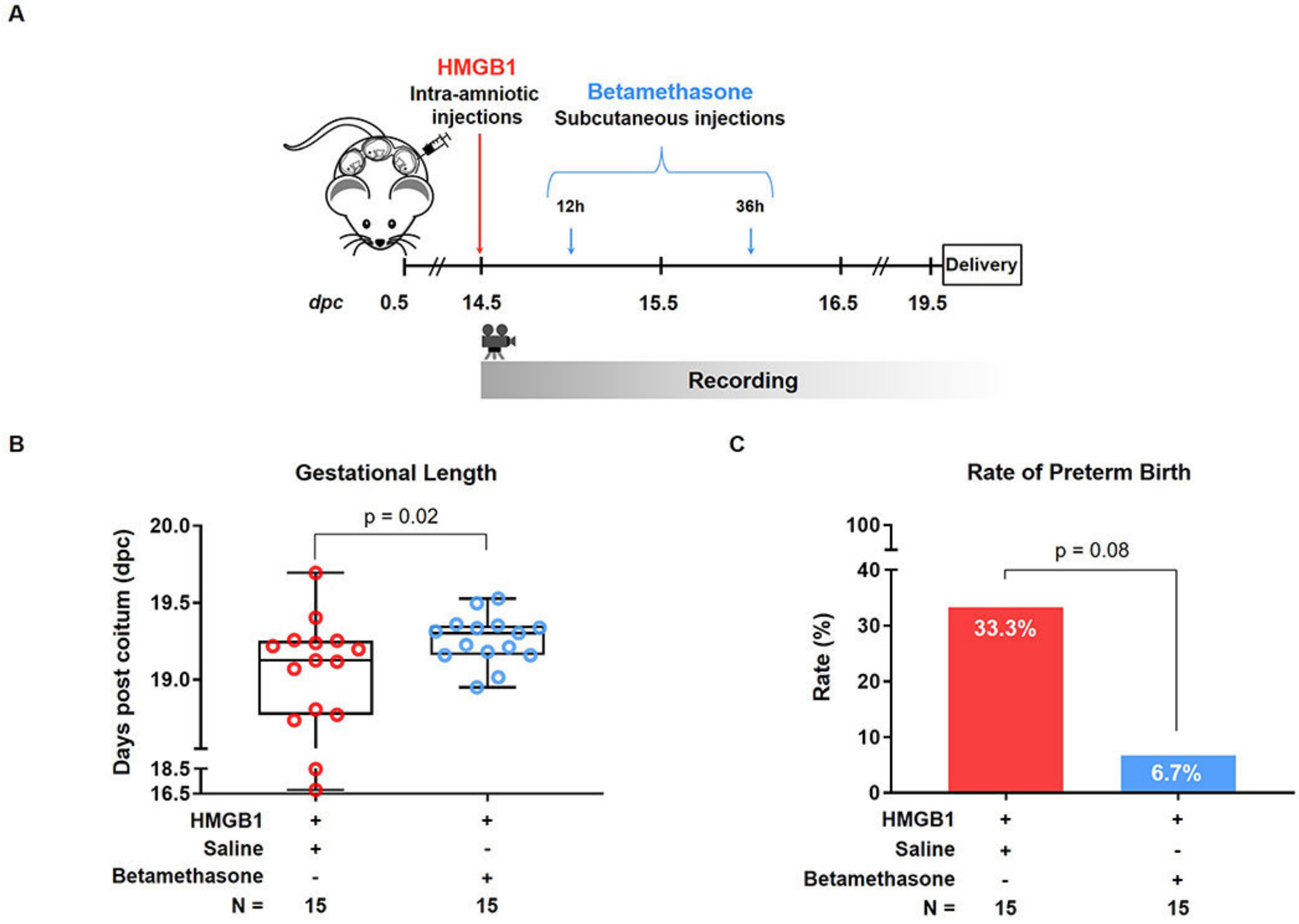

Figure 2. Betamethasone treatment after intra-amniotic injection of HMGB1.

(A) Experimental design: Dams were intra-amniotically injected with HMGB1 and subcutaneously treated with betamethasone (0.1 mg/100 μL) or vehicle control (saline, 100 μL) at 12 and 36 hours (h) after intra-amniotic injection, and monitored until delivery (n = 15 each). **(B)** Gestational length [shown as days *post coitum* (dpc)] of dams injected with HMGB1 and treated with vehicle (saline, red dots) or betamethasone (blue dots). Data are shown as boxplots where the midline represents the median, boxes represent interquartile range, and whiskers represent the minimum/maximum range. **(C)** Preterm birth rate (<19 dpc) in dams that received HMGB1 and were treated with vehicle (saline, red bar) or betamethasone (blue bar).

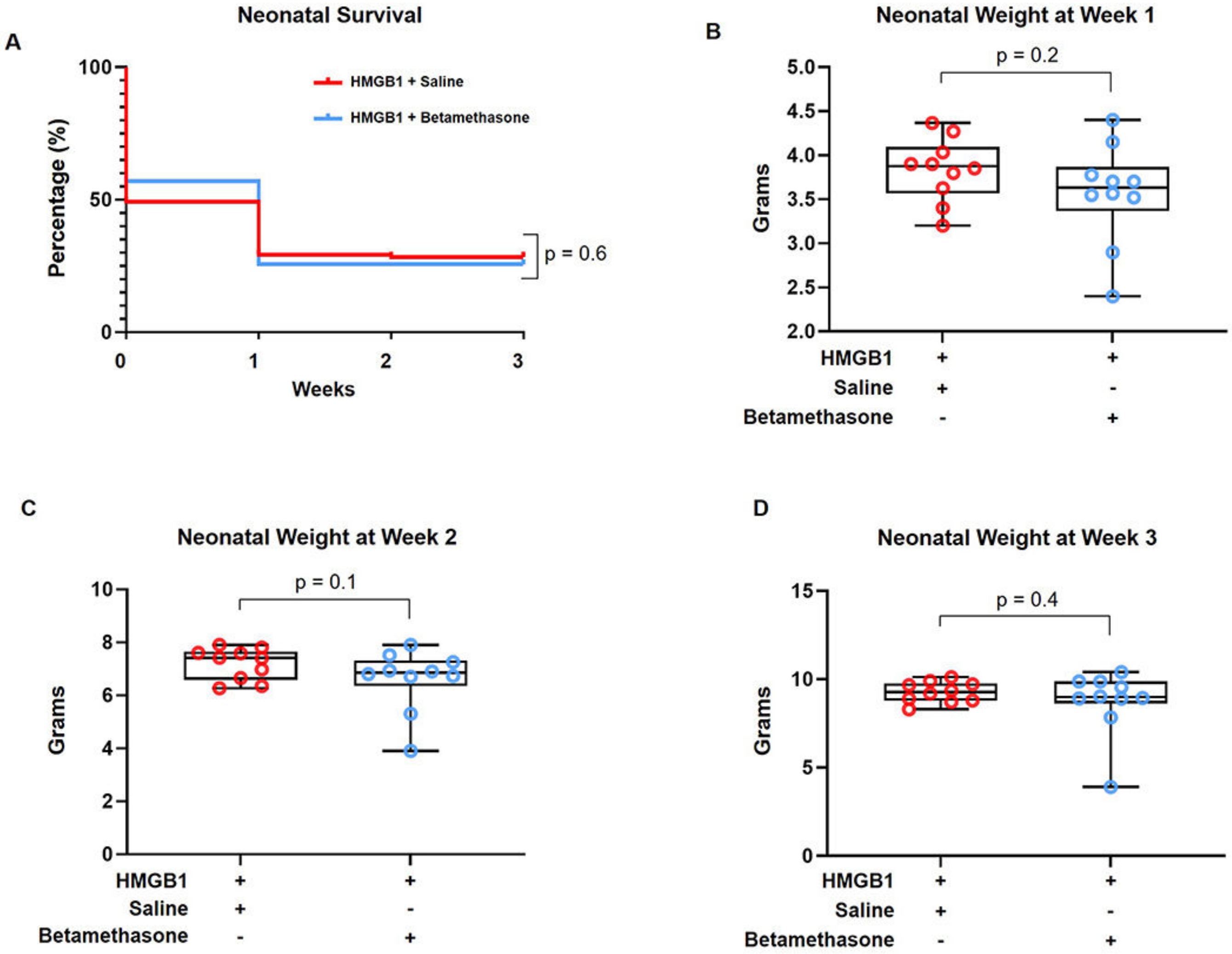

Figure 3. Neonatal outcomes after betamethasone treatment of HMGB1-injected dams

(A) Kaplan-Meier survival curves representing neonatal survival at weeks 1, 2, and 3 postpartum for neonates from dams injected with HMGB1 and treated with vehicle (saline, red line) or betamethasone (blue line) (n = 116 - 120 per group). **(B-D)** Weights of neonates from dams that received HMGB1 and were treated with vehicle (saline, red dots) or betamethasone (blue dots) at weeks 1 **(B)**, 2 **(C)**, and 3 **(D)** postpartum (n = 10 litters each). Data are shown as boxplots where the midline represents the median, boxes represent interquartile range, and whiskers represent the minimum/maximum range. Each dot corresponds to the mean weight of all neonates from a single dam (litter).