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HSP70: An alarmin that does not induce high rates of preterm birth but does cause adverse neonatal outcomes

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Abstract

Objective: Preterm labor and birth are strongly associated with sterile intra-amniotic inflammation, a clinical condition that is proposed to be initiated by danger signals or alarmins. The aim of this study was to investigate whether the intra-amniotic administration of the alarmin heat shock protein 70 (HSP70) induces preterm labor/birth and adverse neonatal outcomes.

Methods: Pregnant mice received an intra-amniotic injection of 200 ng (n = 8), 400 ng (n = 6), 500 ng (n = 10) or 1 μg of HSP70 (n = 6). Control mice were injected with saline (n = 10). Following injection, the rate of preterm labor/birth and neonatal mortality were recorded. Neonatal weights at weeks 1, 2, and 3 were also recorded.

Results: The intra-amniotic injection of 400 ng [late preterm birth 16.7 ± 16.7% (1/6)], 500 ng [early and late preterm birth 10 ± 10% (1/10) each], or 1 μg [early preterm birth 16.7 ± 16.7% (1/6)] of HSP70 induced low rates of preterm/birth. However, the intra-amniotic injection of 500 ng or 1 μg of HSP70 induced significantly higher rates of neonatal mortality compared to controls [saline 14.2% (10/74), 200 ng 9.8% (6/61), 400 ng 17.9% (9/45), 500 ng 28.8% (23/78), and 1 μg 21.4% (13/49)]. Neonates born to dams injected with 200 ng, 500 ng, or 1 μg HSP70 were leaner than controls ($p \leq 0.05$).

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Conflict of Interests

The authors declare no potential conflicts of interest.

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Conclusion: Intra-amniotic administration of the alarmin HSP70 did not induce high rates of preterm labor/birth; yet, it did indeed result in adverse neonatal outcomes.

Keywords

Acute histologic chorioamnionitis; sterile intra-amniotic inflammation; DAMPs; inflammation; parturition; pregnancy; prematurity; funisitis; fetal inflammatory response; amniotic fluid; mouse

Introduction

Preterm birth, a leading cause of perinatal morbidity and mortality worldwide [1, 2, 3, 4], is preceded by spontaneous preterm labor, a syndrome arising from multiple distinct etiologies [5]. Currently, the only established causal link to preterm labor is intra-amniotic infection and inflammation [6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. The majority of women who undergo spontaneous preterm labor with intra-amniotic inflammation do not have detectable microorganisms in amniotic fluid (using both cultivation and molecular microbiology techniques), referred to as sterile intra-amniotic inflammation [18, 19, 20, 21, 22]. This clinical condition is also frequently observed in women with a short cervix [23] and those with clinical chorioamnionitis at term [24]. Patients with preterm labor and sterile intra-amniotic inflammation present similar rates of preterm birth and adverse neonatal outcomes compared to those with intra-amniotic infection [19], highlighting the importance of identifying the initiators of sterile intra-amniotic inflammation, as well as the mechanisms implicated in this clinical condition.

The process of sterile inflammation is initiated by endogenous danger signals, termed damage-associated molecular patterns (DAMPs) [25, 26], or alarmins [27] derived from damaged and necrotic cells. Multiple alarmins are elevated in amniotic fluid of women with preterm labor and intra-amniotic inflammation [28], including interleukin (IL)-1α [29], high-mobility group box-1 (HMGB1) [30], S100B [31], and heat shock protein 70 (HSP70) [32]. Indeed, the administration of IL-1α [33, 34], HMGB1 [35], or S100B [36] induces preterm birth in mice, providing further evidence that alarmins are implicated in the mechanisms that lead to preterm parturition in the context of sterile intra-amniotic inflammation. Yet, whether the exogenous administration of HSP70 can also induce preterm birth has not been shown.

Heat shock proteins (HSPs) are highly conserved molecules [37, 38] that function as molecular chaperones [39, 40, 41, 42] to maintain cellular homeostasis. They are found in almost all subcellular compartments of all cell types and are upregulated in response to a range of physiological, pathological, and environmental stressors including hormonal stimulation, hypoxia, ischemia, and high temperature [43, 44, 45]. Moreover, HSPs participate in innate and adaptive immune responses and their extracellular presence reflects tissue damage or “danger signals” [46]. It has been proposed that extracellular HSPs, released either through non-classical pathways or from necrotic cells [47, 48], act like alarmins, activating monocytes [49, 50, 51] and inducing the secretion of pro-inflammatory cytokines [52, 53, 54]. Specifically, HSP70 concentrations are increased in amniotic fluid of women with spontaneous preterm labor/birth and microbial invasion of the amniotic cavity

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(MIAC) (i.e. intra-amniotic infection) [32]. In the current study, we investigated whether the ultrasound-guided intra-amniotic administration of HSP70 induces preterm labor/birth and adverse neonatal outcomes.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Sacramento, CA) and bred in the animal care facility at C.S. Mott Center for Human Growth and Development at Wayne State University (Detroit, MI). All mice were kept under a circadian cycle (light:dark = 12:12 h). Females, 8-12 weeks old, were bred with males of proven fertility. Female mice were checked daily between 8:00 a.m. and 9:00 a.m. for the appearance of a vaginal plug, which indicated 0.5 days *post coitum* (dpc). Females were then housed separately from the males, their weights were monitored daily, and a gain of two or more grams by 12.5 dpc confirmed pregnancy. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University (Protocol No. A 07-03-15 and 18-03-0584).

Intra-amniotic administration of HSP70

Pregnant C57BL/6 mice were anesthetized on 16.5 dpc by inhalation of 2-3% isoflurane (Aerrane, Baxter Healthcare Corporation, Deerfield, IL, USA) and 1-2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.75-2% isoflurane and 2.0 L/min of oxygen during the ultrasound procedure, which was performed using the Vevo® 2100 Imaging System (VisualSonics Inc., Toronto, Ontario, Canada). Mice were positioned on a heating pad and stabilized with adhesive tape. Fur removal from the abdomen was accomplished by applying Nair depilatory cream (Church & Dwight Co., Inc., Ewing, NJ, USA) to that area. Body temperature was maintained at 37±1°C and detected using a rectal probe (VisualSonics Inc.). Respiratory and heart rates were monitored by electrodes embedded in the heating pad. An ultrasound probe was anchored and mobilized with a mechanical holder, and the transducer was slowly moved toward the Aquasonic CLEAR® ultrasound gel (Parker Laboratories, Inc, Fairfield, NJ, USA) applied on the abdomen. Ultrasound-guided intra-amniotic injection of recombinant human heat shock protein 70 (HSP70; abcam, Cambridge, UK) at concentrations of 200 ng, 400 ng, 500 ng or 1 μg/25 μL of sterile 1X phosphate-buffered saline (PBS; Fisher Scientific Bioreagents, Fair Lawn, NJ, USA) was performed in each amniotic sac using a 30-gauge needle (BD PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ, USA) (n = 6-10). Controls were injected with 25 μL of PBS (n = 10). The syringe was stabilized with a mechanical holder (VisualSonics Inc.). Following the ultrasound, mice were placed under a heat lamp until they regained full motor function, which occurred 5-10 min after heating.

Video monitoring of pregnancy outcomes

Immediately after intra-amniotic injection of HSP70 or PBS, dams were monitored until delivery using a video camera and infrared light (Sony Corporation, Tokyo, Japan). Gestational length was defined as the time elapsed from the detection of the vaginal plug (0.5 dpc) through the delivery of the first pup. Preterm labor/birth was defined as

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delivery occurring before 18.5 dpc, and its rate was represented by the percentage of females delivering preterm among the total number of mice. We classified preterm birth in two distinct groups; early preterm birth which was defined as delivery before day 18.0 of gestation, and late preterm birth which was defined as delivery between day 18.0 and 18.5 of gestation. The rate of neonatal mortality for each litter was defined as the proportion of delivered pups found dead among the total litter size. Neonatal weights at weeks 1, 2, and 3 were also recorded.

Statistical analysis

Data analysis was completed using SPSS version 19.0 software (IBM Corp, Armonk, NY, USA). Differences in the rate of preterm birth between the control group (i.e. PBS) and study groups, as well as the rate of pup mortality at birth, were analyzed using the Fisher's exact test. The neonatal weights at weeks 1, 2, and 3 were compared between control and study groups using the Mann-Whitney U test. A p-value ≤ 0.05 was regarded as statistically significant for all tests.

Results

Intra-amniotic administration of high concentrations of HSP70 induces low rates of preterm birth

Pathophysiological concentrations of HSP70 in the amniotic fluid range from 80 ng to 500 ng [32]. First, we intra-amniotically injected pregnant mice with elevated HSP70 concentrations ranging from 200 ng to 500 ng (Figure 1A). Dams injected with PBS (controls) or 200 ng of HSP70 delivered at term (Figure 1B). However, a small proportion of dams injected with 400 ng of HSP70 delivered late preterm [16.7 ± 16.7% (1/6)] (Figure 1B). Higher concentrations of HSP70 (500 ng) induced both early and late preterm birth [10 ± 10% (1/10) each] (Figure 1B); yet, the effect was still minimal. Given that we have previously shown that mice require double the pathological amniotic fluid concentration of LPS [55] and S100B [31] to deliver preterm [36, 56], we decided to double the concentration of HSP70 to 1 μg. However, the intra-amniotic injection of 1 μg of HSP70 also induced low rates of early preterm birth [16.7 ± 16.7% (1/6)] (Figure 1B). These results show that the intra-amniotic administration of the alarmin HSP70 induces low rates of preterm birth, even at supraphysiological concentrations.

Intra-amniotic administration of HSP70 causes adverse neonatal outcomes

The intra-amniotic injection of HSP70 did not induce high rates of preterm birth; however, elevated intra-amniotic concentrations of this alarmin may be inducing fetal damage. Therefore, we evaluated the rate of neonatal mortality after intra-amniotic administration of HSP70. Most neonates born to dams injected with PBS (controls), 200 ng, or 400 ng of HSP70, delivered at term and thrived up to 3 weeks of life [14.2 ± 4.8% (10/74), 9.8 ± 5.4% (6/61), and 17.9 ± 8.9% (9/45), respectively] (Figure 2). However, some of the neonates born to dams injected with 500 ng or 1 μg of HSP70 died at birth [28.8 ± 12.9% (23/78) and 21.4 ± 16.0% (13/49), respectively] (Figure 2).

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Lastly, we assessed the wellbeing of the fetus by determining the neonatal weight at weeks 1, 2, and 3. At one week of age, no differences in weight were found between the HSP70 groups (regardless of the concentration) and controls (Figure 3). However, neonates born to dams injected with 200 ng, 500 ng, or 1 μg of HSP70 were leaner than those born to control dams at two weeks of age [200 ng HSP70: median 6.4 g (IQR = 5.65–7.1 g) versus PBS: median 7.1 g (IQR = 6.6–7.65 g); $p = 0.017$], [500 ng HSP70: median 6.7 g (IQR = 6.2–7.2) versus PBS: median 7.1 g (IQR = 6.6–7.65 g); $p = 0.05$] or [1 μg HSP70: median 6.6 g (IQR = 5.8–7.1) versus PBS: median 7.1 g (IQR = 6.6–7.65 g); $p = 0.006$] (Figure 3). No differences were observed in the neonatal weights among the study groups at the third week of age (Figure 3), suggesting that thriving neonates recover from the effects of HSP70 by the third week.

Together, these data indicate that the intra-amniotic administration of HSP70 at supraphysiological concentrations impacts neonatal life.

Discussion

Herein, we report that, while intra-amniotic injection of HSP70 did not induce preterm birth to a significant degree, it did indeed result in adverse neonatal outcomes. These findings show that HSP70 has a milder intra-amniotic effect than previously studied alarmins (i.e. HMGB1 [35] and S100B [36]).

The alarmin HSP70 can induce adverse pregnancy outcomes in the context of infection, sterile inflammation, and oxidative stress, among others [57, 58]. HSP70 is recognized by the pattern recognition receptors (PRRs) TLR2 and TLR4 [59, 60], which are expressed in the chorioamniotic membranes [61], placenta [62, 63, 64], and other fetal tissues [65]. The engagement of TLR2 and TLR4 by HSP70 leads to activation of the nuclear factor kappa B (NF-κB) pathway, resulting in the release of pro-inflammatory cytokines such as TNF-α, IL1-β, and IL-6 [52]. NF-κB also serves as an inhibitor of autophagy [66], and the expression of proteins associated with this mechanism of cell death are altered in the placenta and chorioamniotic membranes of women who underwent spontaneous preterm labor [67, 68], suggesting that HSP70 is implicated in the inflammatory process of preterm parturition. In addition to triggering the NF-κB pathway, HSP70 can lead to the activation of mTORC1 [69], which is a major inhibitor of autophagy [70, 71, 72]. The combined inhibition of autophagy by NF-κB and mTORC1 results in an increase of intracellular reactive oxygen species (ROS) and pro-inflammatory cytokines [58], which suggests that HSP70 may induce intra-amniotic inflammation through the inhibition of autophagy.

In the current study, low rates of preterm birth were observed after intra-amniotic injection of pathological concentrations of HSP70 [32] (400 ng to 1 μg). A potential explanation for this finding is that the sole administration of HSP70 induces a mild inflammatory response that is not severe enough to cause high rates of preterm birth. This is in contrast to what occurs in the clinical scenario of intra-amniotic infection, where microorganisms invading the amniotic cavity initiate strong inflammatory responses [73, 74, 75, 76], resulting in high amniotic fluid concentrations of HSP70 [32]. This concept is supported by previous studies showing that the chorioamniotic membranes displayed increased mRNA expression

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of HSP70 upon incubation with endotoxin [77]. In this setting, alarmins such as HSP70 could be released by necrotic and apoptotic cells [48] that are generated upon treatment with endotoxin or other microbial products [78, 79].

Nonetheless, our findings showed that adverse neonatal outcomes did occur upon intra-amniotic injection of HSP70. This suggests that, while HSP70 alone is not sufficient to induce high rates of preterm birth, the fetal inflammatory response initiated by this alarmin could result in adverse neonatal outcomes, which are similar to those observed in neonates born to dams injected with HMGB1 or S100B [35, 36]. Such a fetal inflammatory response is more severe in cases of intra-amniotic infection, causing adverse neonatal outcomes [80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97]. In line with this concept, elevated systemic concentrations of extracellular HSP70 have been associated with other inflammatory diseases [98, 99, 100]. Although more investigation is required to investigate the fetal inflammatory responses induced by HSP70, it is tempting to suggest that this alarmin is sensed by PRRs expressed on innate and adaptive immune cells present in the amniotic cavity [101, 102, 103, 104, 105, 106].

In summary, the findings herein provide evidence that the alarmin HSP70 can induce adverse neonatal outcomes without necessarily inducing premature labor and birth. These data show that not all alarmins in the amniotic cavity are sufficient to cause preterm labor/birth, yet these inflammatory mediators may still impact neonatal quality of life. Further research is needed in order to investigate the mechanisms whereby HSP70 in the amniotic cavity induces adverse neonatal outcomes.

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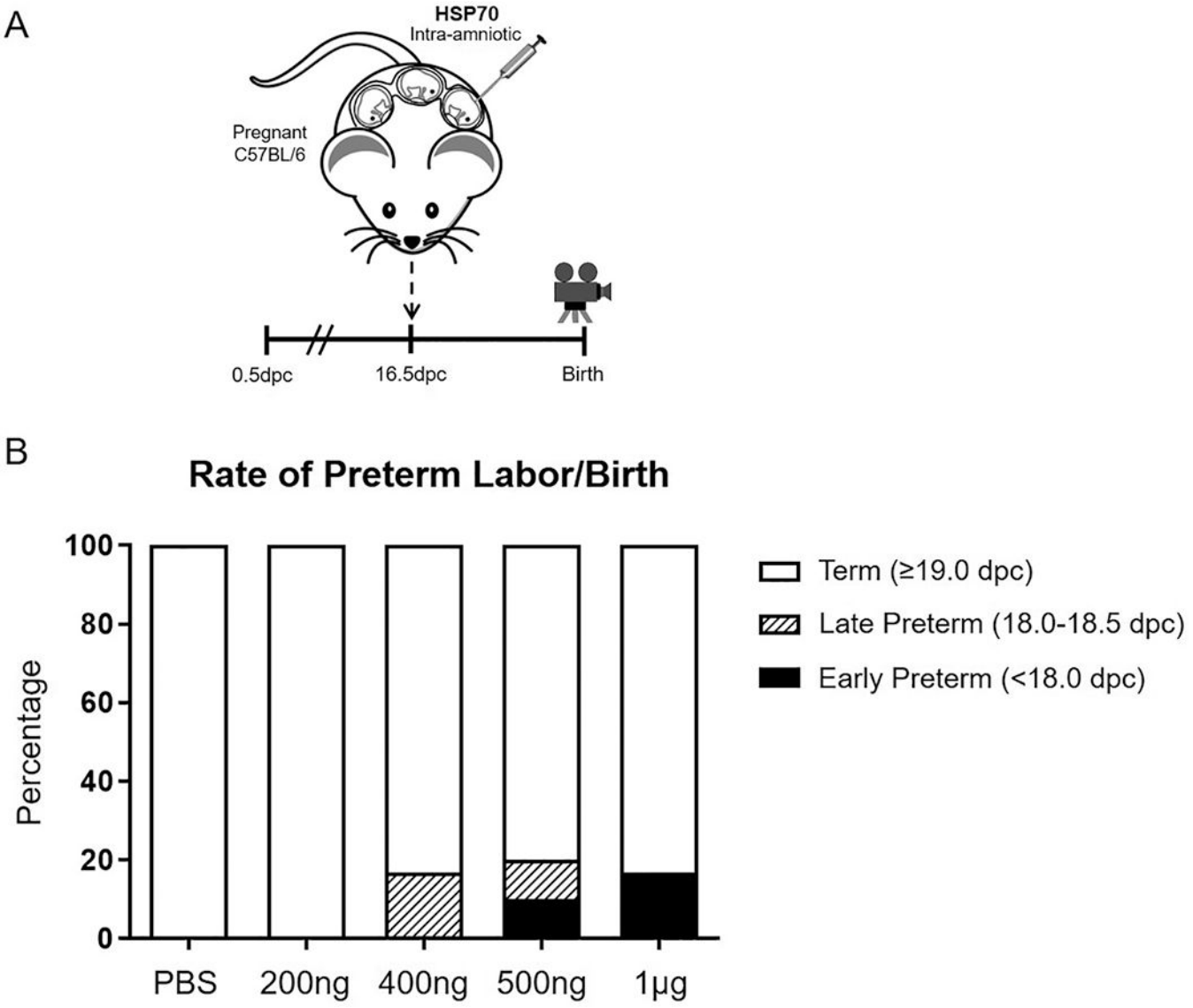

Figure 1. Intra-amniotic administration of the alarmin HSP70.

(A) Pregnant C57BL/6 dams were intra-amniotically injected with heat shock protein 70 (HSP70) or saline (1X phosphate-buffered saline; PBS) on 16.5 days post coitum (dpc). **(B)** The rate of preterm birth of mice injected intra-amniotically with 200 ng/25 µL, 400 ng/25 µL, 500 ng/25 µL, 1 µg/25 µL, or saline. The black bars represent early preterm birth (<18.0 dpc) and the striped bars represent late preterm birth (18.0-18.5 dpc). N = 6-10 dams per group.

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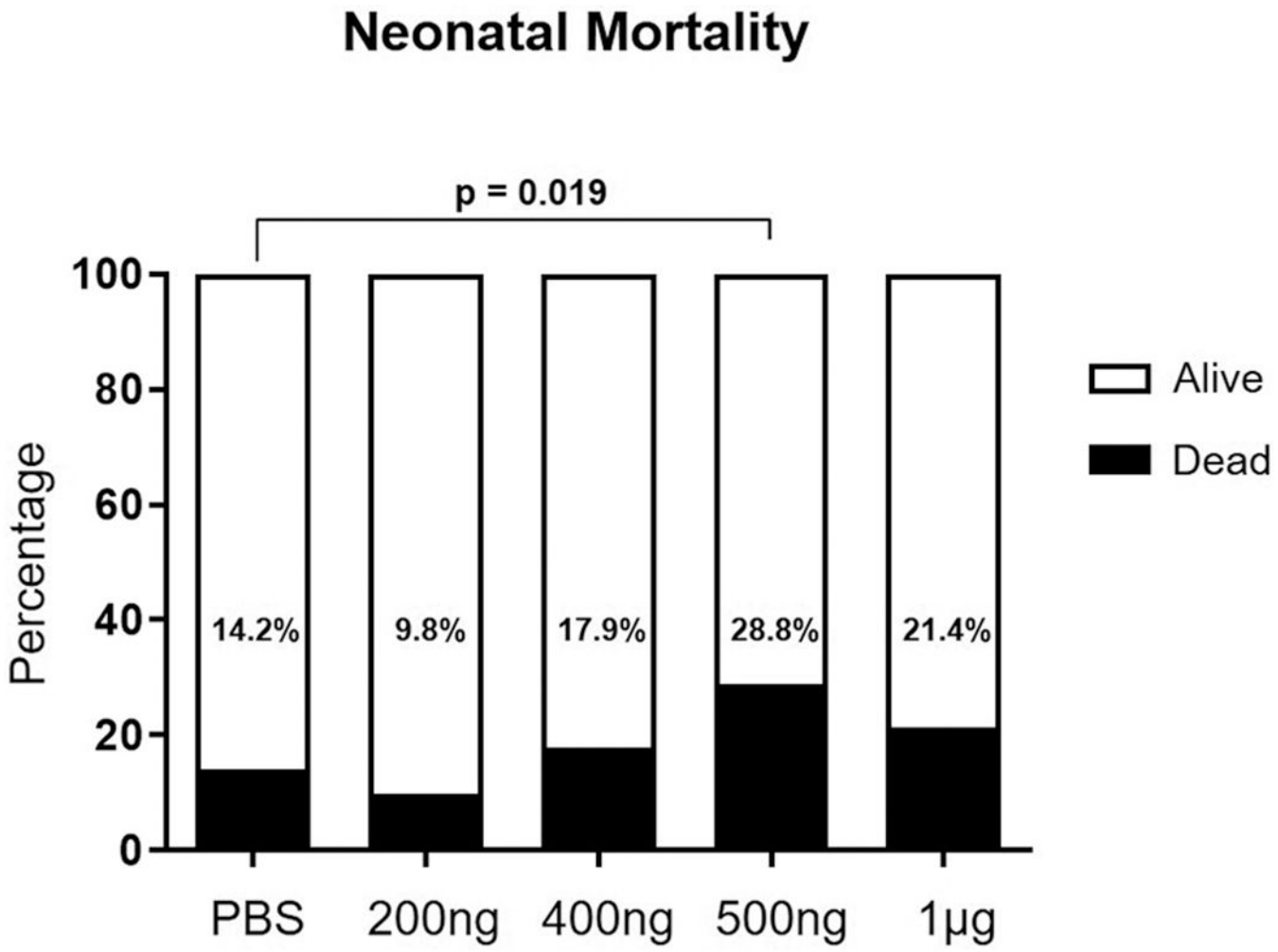

Figure 2. The rate of neonatal mortality.

Pregnant mice were injected intra-amniotically with 200 ng/25 µL, 400 ng/25 µL, 500 ng/25 µL, 1 µg/25 µL, or saline (1X phosphate-buffered saline; PBS) and the rate of neonatal mortality was recorded at birth. N = 6-10 dams per group.

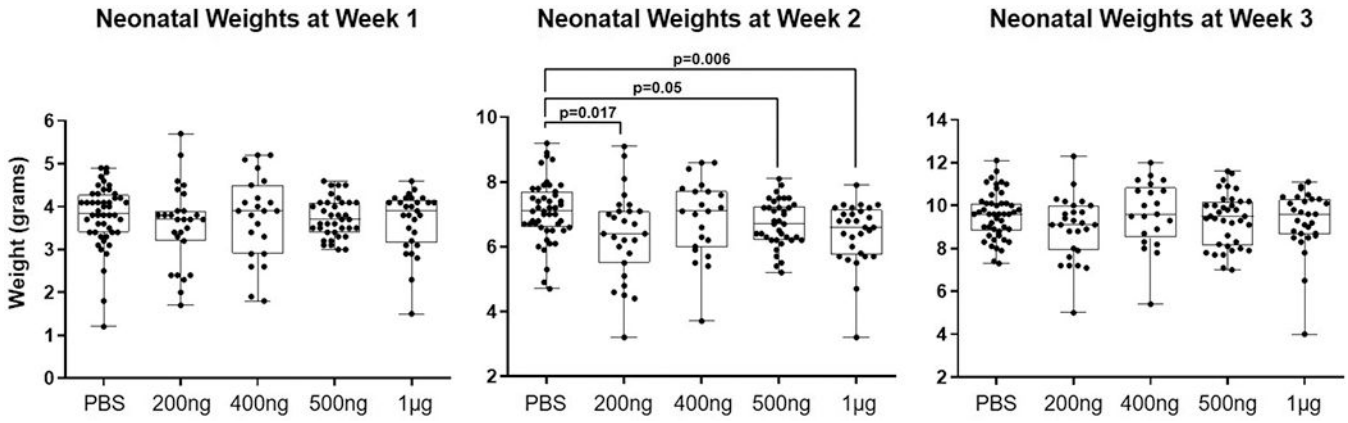

Figure 3. Neonatal weights at weeks 1, 2, and 3 after birth.

Neonates were weighed at the ages of 1 week, 2 weeks, and 3 weeks. N = 4-9 litters per group.

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