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Intra-amniotic Administration of HMGB1 Induces Spontaneous Preterm Labor and Birth

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ABSTRACT

Problem: Sterile intra-amniotic inflammation is associated with spontaneous preterm labor. Alarmins are proposed to mediate this inflammatory process. The aim of this study was to determine whether intra-amniotic administration of an alarmin, HMGB1, could induce preterm labor/birth.

Method of Study: Pregnant B6 mice were intra-amniotically or intraperitoneally injected with HMGB1 or PBS (control). Following injection, the gestational age and the rates of preterm birth and pup mortality were recorded.

Results: Intra-amniotic injection of HMGB1 led to preterm labor/birth [HMGB1 57% (4/7) *vs.* PBS 0% (0/6); p=0.049), and a high rate of pup mortality at week one [HMGB1 60.9±11.7% (25/41) *vs.* PBS 28.9±12.6% (11/38); p=0.001)

Conclusion: Intra-amniotic administration of HMGB1 induces preterm labor/birth.

INTRODUCTION

Preterm birth, or birth occurring prior to the 37th week of gestation, is the leading cause of perinatal morbidity and mortality. Approximately 70% of all preterm births are preceded by spontaneous preterm labor,[1] a syndrome of caused by multiple pathological processes.[2] Of all the putative causes associated with spontaneous preterm labor, only intra-amniotic infection/inflammation has been causally linked to preterm birth.[2] Sterile intra-amniotic inflammation, an inflammatory process (interleukin (IL)6 $\geq$ 2.6 ng/mL) in the absence of microorganisms, is more common than microbial-associated intra-amniotic inflammation in patients with preterm labor and intact fetal membranes.[3] Sterile intra-amniotic inflammation is also frequently observed in patients with a sonographic short cervix,[4] and in those with preterm prelabor rupture of the membranes and clinical chorioamnionitis.[5] The inflammatory process in sterile inflammation results from activation of the innate immune system by endogenous danger signals, derived from necrosis or cellular stress,[6] termed damage-associated molecular pattern molecules (DAMPs)[7] or alarmins.[8] Since the concentration of several alarmins, including IL1α,[9] S100 calcium-binding protein B,[10] heat shock protein 70,[11] and high-mobility group box-1 (HMGB1),[12, 13] is increased in the amniotic fluid of women with intra-amniotic inflammation, we proposed that these danger signals are responsible for sterile inflammation.[11-13]

HMGB1 is an evolutionarily conserved protein that stabilizes nucleosome formation and facilitates gene transcription while localized to the nucleus; however, it acts as an alarmin when released extracellularly.[14] HMGB1 demonstrates the four classic characteristics of an alarmin: 1) rapid release following non-programmed cell death (i.e., necrosis) but not as a result of apoptosis; 2) production and release by viable immune cells through specialized secretion systems or the endoplasmic reticulum-Golgi secretion pathway; 3) recruitment and activation of innate immune cells via pattern recognition receptors (PRR) which, in turn, can directly or indirectly promote adaptive immune responses; and 4) restoration of homeostasis through the healing of tissue directly or indirectly damaged by inflammation.[15]

Patients with sterile intra-amniotic inflammation and a high concentration of HMGB1 in the amniotic fluid deliver earlier than those with a low concentration of this alarmin.[3] In addition, HMGB1 concentrations in the maternal serum are elevated in pregnancies with reduced fetal movement.[16] Therefore, we hypothesized that intra-amniotic, but not systemic, administration of

HMGB1 would induce preterm birth. We tested this hypothesis by injecting pregnant B6 mice with HMGB1 intra-amniotically or intraperitoneally.

MATERIALS AND METHODS

Animals

C57BL/6 (B6) mice were purchased from The Jackson Laboratory in Bar Harbor, ME, USA, and bred in the animal care facility at the C.S. Mott Center for Human Growth and Development at Wayne State University, Detroit, MI, USA. All mice were kept under a circadian cycle (light:dark = 12:12h). Females, 8-12 weeks old, were mated with males of the same phenotype. 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 weight was monitored, 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 09-08-12).

Intra-Amniotic Administration of HMGB1

Pregnant B6 mice were anesthetized on 14.5 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 1.5-2% isoflurane and 1.5-2 L/min of oxygen. Mice, positioned on a heating pad, were stabilized with adhesive tape. Fur removal from the abdomen and thorax occurred after the application of Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA) to the area. Body temperature was maintained in the range of 37±1˚C and detected with a rectal probe. Respiratory and heart rates were monitored by electrodes embedded in the heating pad. An ultrasound probe was fixed and mobilized with a mechanical holder, and the transducer was slowly moved toward the abdomen. Ultrasound-guided intra-amniotic injection of endotoxin-free HMGB1 (IBL International Corp., Toronto, ON, CA) at a concentration of 9 ng^3 dissolved in 100 µL of sterile 1X phosphate-buffered saline (PBS; Fisher Scientific Bioreagents, Fair Lawn, NJ, USA; n=7) or in PBS (100 µL; control; n=6) was performed in each amniotic sac using a 30 G x ½ in (0.3 mm x 25 mm) needle (BD

PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ, USA) (Figure 1A). The syringe was stabilized by a mechanical holder (VisualSonics Inc). Intra-amniotic injections on 15.5 dpc and 16.5 dpc were not always feasible due to the low volume of amniotic fluid. Following ultrasound, mice were placed under a heat lamp for recovery, which occurred 10-20 min after heating. On the evening of 17.5 dpc, mice were monitored via video recording using an infrared camera (Sony Corporation, China) in order to determine gestational age and the rates of preterm birth, and pup mortality at birth and week one.

Intraperitoneal Administration of HMGB1

Pregnant B6 mice were intraperitoneally injected with endotoxin-free HMGB1 on 16.5 dpc (Figure 2A). The concentration of HMGB1 in serum in cases of reduced fetal movement ranges from 10 ng/mL to 50 ng/mL.[16] However, these concentrations are not associated with spontaneous preterm labor.[16] The *in vivo* effects of HMGB1 are observed only when a greater amount (10 - 100µg per mouse) is intraperitoneally injected.[17] Therefore, two different concentrations were tested: 20 µg (n=4) and 50 µg (n=10) dissolved in 200 µL of PBS. Control mice were injected with 200 µL of PBS (n=10). Following injection, mice were monitored via video recording using an infrared camera (Sony Corporation) in order to determine gestational age and the rates of preterm birth, and pup mortality at birth and week one.

Outcome Variables

Gestational age was defined as the time elapsed from the detection of the vaginal plug (0.5 dpc) through the delivery of the first pup. The rate of pup mortality at birth was defined as the percentage of pups found dead among the total litter size. Preterm birth was defined as a delivery occurring before or on 18.5 dpc, and its rate was represented by the percentage of females delivering preterm among those delivering at term (19.5 ± 0.5 dpc). The rate of pup mortality at week one was defined as the number of pups that died before one week of age among the total number of pups born alive.

Statistical Analysis

Statistical analyses were performed using SPSS, Version 19.0 (IBM Corporation, Armonk, NY, USA). The following tests were performed to compare differences between the groups: the Mann-Whitney U test for gestational age, a t-test for pup weight, a Fisher's exact test for the rates of preterm birth, and a logistic regression model for the rates of pup mortality. A p value of 0.05 was considered statistically significant.

RESULTS

The frequency of preterm labor/birth was higher after an intra-amniotic injection of HMBG1 (9 ng/100µL) than following the intra-amniotic injection of PBS [HMGB1 57% (4/7) *vs.* PBS 0% (0/6); p=0.049; Figure 1B]. Pregnant mice injected with HMGB1 had a shorter gestational age than mice injected with PBS (HMGB1 18.7±0.3 dpc *vs.* PBS 19.45±0.3 dpc; p=0.001; Figure 1C). Intra-amniotic injection of HMGB1 was associated with a modest increase in pup mortality at birth, but this did not reach statistical significance [HMGB1 14.5±9.3% (7/48) *vs.* PBS 9.5±8.4% (4/42); Figure 1D]. In addition, intra-amniotic injection of HMGB1 was associated with an increased rate of pup death by the age of one week [HMGB1 60.9±11.7% (25/41) *vs.* PBS 28.9±12.6% (11/38); p=0.001; Figure 1E]. No differences between groups were observed in pup weight (Figure 1F).

Intraperitoneal injection of HMGB1 [20µg/200µL (data not shown) or 50 µg/200µL] did not induce preterm labor/birth [HMGB1 0% (0/10) *vs.* PBS 0% (0/10); Figure 2B], and all injected mice delivered at term (Figure 2B and 2C). Intraperitoneal injection of HMBG1 had no effect on the pup viability at birth since most of the pups were born alive (Figure 2D). However, intraperitoneal injection of HMGB1 increased the rate of pup mortality at week one [HMGB1 32.4±9.4% (23/71) *vs.* PBS 13±7.4% (9/69); p=0.01; Figure 2E]. No differences between groups were observed in pup weight (Figure 2F).

DISCUSSION

The study herein demonstrates that intra-amniotic administration of HMGB1, given in doses similar to that observed in the amniotic fluid of patients with sterile intra-amniotic inflammation who underwent spontaneous preterm labor,[3] can result in preterm labor/birth in mice. In contrast, intraperitoneal injection of HMGB1 at an even higher concentration does not cause preterm labor/birth. This finding provides evidence that an alarmin -HMGB1- can induce

premature labor, and therefore may be involved in signaling parturition in the context of sterile intra-amniotic inflammation.[3-5]

The etiology of sterile intra-amniotic inflammation is unknown; yet, this clinical condition has been associated with an elevated concentration of HMGB1 in the amniotic fluid.[3] Herein, we demonstrated that intra-amniotic injection of HMGB1 induces preterm birth, whereas intraperitoneal injection at a much higher concentration (an increase of ~300 or 790-fold per mouse) failed to produce the same effect. The effect of HMGB1 is probably mediated through action on the amnion, as HMGB1 is strongly immunolocalized in amnion epithelial cells but weakly present in the chorioamniotic connective tissue layer and infiltrating leukocytes.[12]

In summary, the data herein show that intra-amniotic injection of HMGB1 can induce preterm labor/birth. This finding supports the concept that alarmins are implicated in the mechanisms of parturition in sterile intra-amniotic inflammation. Further research is needed in order to investigate the immune mechanisms whereby HMGB1 in the amniotic cavity induces spontaneous preterm labor.

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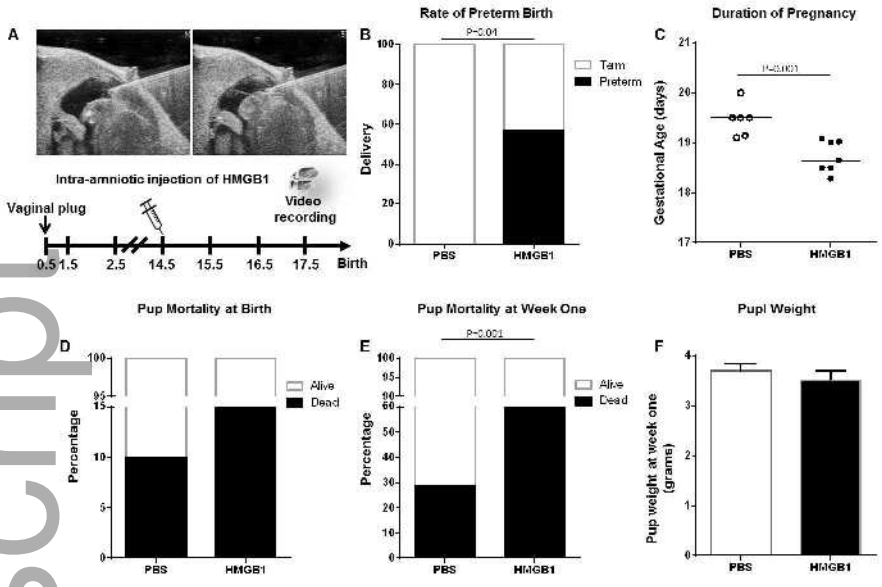
FIGURE LEGENDS

Figure 1. Intra-amniotic injection of HMGB1. A) On 14.5 dpc, pregnant mice were intra-amniotically injected with HMGB1 (9 ng/100 µL; n=7) or PBS (100 µL; n=6), using 3D ultrasound, and mice were video-monitored until delivery. (B) Rate of preterm birth. (C) Gestational age. (D) Rate of pup mortality at birth. (E) Rate of pup mortality at week one. (F) Pup weight.

Figure 2. Intraperitoneal injection of HMGB1. A) On 16.5 dpc, pregnant mice were intraperitoneally injected with HMGB1 (50 µg/200 µL; n=10) or PBS (200 µL; n=10) and video-monitored until delivery. (B) Rate of preterm birth. (C) Gestational age. (D) Rate of pup mortality at birth. (E) Rate of pup mortality at week one. (F) Pup weight.



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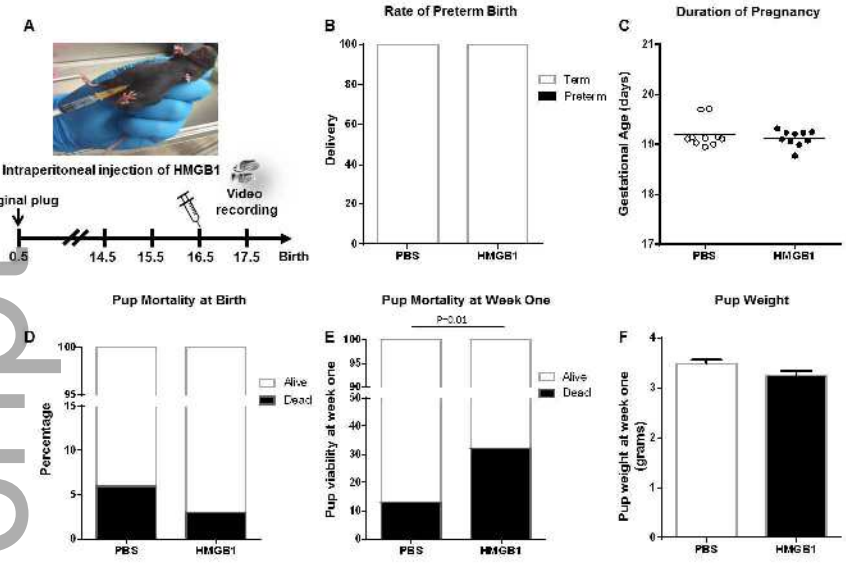

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