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Combined *in utero* hypoxia-ischemia and lipopolysaccharide administration in rats induces chorioamnionitis and a fetal inflammatory response syndrome

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ABSTRACT

Introduction: Preterm birth is a major cause of infant morbidity and long-term disability, and is associated with numerous central nervous system (CNS) deficits. Infants exposed to intrauterine inflammation, specifically chorioamnionitis, are at risk for very early preterm birth and neurological complications including cerebral palsy, epilepsy, and behavioral and cognitive deficits. However, placenta-brain axis abnormalities and their relationship to subsequent permanent CNS injury remain poorly defined.

Methods: Intrauterine injury was induced in rats on embryonic day 18 (E18) by transient systemic hypoxia-ischemia (TSHI) and intra-amniotic lipopolysaccharide (LPS) injection. Placenta, brain and serum were collected from E19 to postnatal day 0 (P0). Histology, TUNEL staining, western blot and multiplex immunoassays were used to quantify placental and brain abnormalities, and fetal serum cytokine levels.

Results: Prenatal TSHI + LPS caused acute and subacute placental injury hallmarked by inflammatory infiltrate, edema, hemorrhage and cell death along with placental increases in IL-1β and TNFα. TSHI + LPS increased a diverse array of circulating inflammatory proteins including IL-1β, TNFα, IL-6, IL-10, IL-4, IFNγ and CXCL1, both immediately after TSHI + LPS and in live born pups. CNS inflammation was characterized by increased CXCL1.

Discussion: Prenatal TSHI + LPS in rats induces placental injury and inflammation histologically consistent with chorioamnionitis, concomitant with elevated serum and CNS pro-inflammatory cytokines. This model accurately recapitulates key pathophysiological processes observed in extremely preterm infants including placental, fetal, and CNS inflammation. Further investigation into the mechanism of CNS injury following chorioamnionitis and the placental-brain axis will guide the use of future interventions.

1. Introduction

Prematurity is a major cause of infant mortality and long-term disability in children [1–4]. Survivors often have numerous neuropsychiatric and neurological disabilities, including poor social interaction, attention deficit hyperactivity disorder and intellectual disability [1–4]. Encompassing extensive effects on neurodevelopment, preterm birth also increases the risk of cerebral palsy, impaired learning, vision and hearing loss, epilepsy, and overall poor physical health that cumulatively contributes to the prematurity-related burden of chronic disease in adulthood [5,6]. Epidemiological, preclinical and clinical studies support a placental-brain axis in neurological development rooted in common cellular and molecular mechanisms [7,8]. To identify and refine targets for novel therapeutic interventions, a clearer understanding of how *in utero* insults translate to impaired

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neurodevelopment is imperative.

Acute chorioamnionitis is the most common histological abnormality found in placentas from extreme preterm infants [9]. Infants exposed to both placental perfusion defects and chorioamnionitis have the highest risk for poor long-term neurological outcomes [4,10–12]. Detailed analyses of placentas from extremely low gestational age newborns (ELGANs) show high-grade inflammation is more commonly associated with spontaneous early birth, whereas less inflammation is present in placentas from conditions such as pre-eclampsia [13]. Chorioamnionitis and the subsequent pathophysiology leads to a disruption of the maternal–fetal interface that affects the fetus by creating an abnormal microenvironment characterized by inflammation, and limited oxygen and nutrient exchange [14,15].

Both prenatal infection and hypoxia-ischemia (HI) induce systemic and central nervous system (CNS) inflammatory signaling. Notably, chorioamnionitis is associated with hemodynamic alterations, indicative of central contributions of both inflammation and ischemia to the pathogenesis of brain injury in the preterm infant [14,15]. Indeed, early hemodynamic disturbances have been shown in preterm newborns with chorioamnionitis and elevated cord blood cytokines or premature rupture of membranes, but not their peers without these signs [14,16]. Detailed study of placental injury in a clinically relevant animal model can provide understanding of the mechanisms involved in fetal systemic inflammation, neuroinflammation and cerebral injury. Given the placenta's unique role as a platform and interface for fetal maturity essential to CNS development, we analyzed placenta, fetal serum and brain using our established preclinical model of prenatal transient systemic hypoxia-ischemia (TSHI) and intra-amniotic lipopolysaccharide (LPS) [17–19]. Previously, we published the individual contributions of TSHI and LPS alone, and combined TSHI + LPS insult to CNS injury [17]. We performed detailed analysis of ventriculomegaly, gliosis, myelination, axonal health, erythropoietin (EPO) and EPOR mRNA expression and gait abnormalities [17]. Notably, prenatal TSHI + LPS leads to significant ventriculomegaly, astro-and microgliosis, and reduced potassium-chloride transporter 2 (KCC2) expression [17,19]. We also demonstrated alterations in myelin basic protein expression, axons, and numerous functional gait abnormalities in stride, paw placement, consistency and coordination in young adult rats [17]. However, the direct impact of this injury on the placenta, the developing fetus and the putative pathophysiologic relationship to brain injury has not yet been investigated. We hypothesized prenatal TSHI plus intra-amniotic LPS would result in placental inflammation consistent with chorioamnionitis and placental disease similar to that observed in human infants born very preterm. Additionally, we predicted that fetal systemic cytokine protein expression would correlate with both placental and early brain inflammation, consistent with a fetal inflammatory response syndrome (FIRS) often observed in human infants born preterm [20].

2. Materials and methods

Institutional Care and Use Committees at Boston Children's Hospital and University of New Mexico Health Sciences Center approved all experimental procedures.

2.1. Transient systemic hypoxia-ischemia and intra-amniotic LPS injections

Pregnant Sprague Dawley rats (Charles Rivers Laboratories, Wilmington, MA) underwent a laparotomy on embryonic day 18 (E18), as described previously [17,19]. Briefly, under anesthesia, uterine arteries are occluded for 60 min with aneurysm clips. After clips are removed, 4 μg of LPS in sterile saline (LPS 0111:B4, Sigma, St. Louis, MO) is injected in to each amniotic sac using an 8 mm 31G needle in a maximum volume of 100 μl/sac. The laparotomy is then closed [17,19]. For sham controls, uterine horns are exposed for 60 min without artery occlusion or LPS injection. Naïve animals did not undergo anesthesia or laparotomy. Dams recover and are monitored until tissue collection. Pups are born at E22. Both sexes are used for all experiments.

2.2. Serum, brain and placenta collection

Placentas were harvested at E19 or E21. Brains were collected from rat pups at E19 or on postnatal day 0 (P0). Tissues were rapidly dissected, flash frozen and stored at −80 °C or fixed in 4% paraformaldehyde. Whole blood was collected and centrifuged at 6000 RCF at 4 °C for 15 min. Serum was collected and stored in aliquots at −80 °C.

2.3. Hematoxylin and eosin (H&E)

E19 and E21 placentas were bisected from the maternal to the fetal surface, embedded in paraffin, and cut at 6 μm. Sections were stained with hematoxylin and eosin (H&E) per standard protocols [17,19].

2.4. Placental injury scoring

Together with an anatomic pathologist (N.J.), we modified a previously published rat placental injury grading scale [21], incorporating key features of human chorioamnionitis including neutrophil infiltration, edema, hemorrhage, infarction and vascularity [22–25]. H&E stained slides were reviewed by a pathologist and scored by two observers blinded to injury condition on a scale of 0–4, with 0 = no pathology, 1 = minimal decidual necrosis, 2 = decidual necrosis along the basal plate, 3 = extensive decidual necrosis with inflammation, microabscesses and parenchymal necrosis, and 4 = severe necrosis throughout the placental layers with variable edema and hemorrhage.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed on a subset of paraffin-embedded slide mounted sections to detect cell death using a POD *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN) as previously reported [26].

2.6. Multiplex determination of cytokine and chemokine expression

Utilizing a V-PLEX immunoassay (MesoScale Discovery, Gaithersburg, MD), the following seven cytokines and chemokines were analyzed in placenta, serum and brain: TNFα, IL-1β, IL-4, IL-6, IL-10, IFNγ and CXCL1 (n = 5–13 serum/group, n = 4–6 placenta/group, n = 7–9 brain/group). Assays were performed similar to studies in humans [27]. Brain or placental lysate (100 μg), serum (1:4 dilution) or calibrator, was loaded onto a multi-spot plate and assay performed per the manufacturer's protocol. Plates were read on a Quickplex SQ 120 Imager (MesoScale Discovery, Gaithersburg, MD). Coefficient of variation was less than 15% for all 7 analytes.

2.7. Western blot

Western blots were performed on placental whole cell fractions at E19 (n = 7–10/group). Protein (30 μg) was equalized and loaded on 4–12% BisTris gels (BioRad, Hercules, CA). Following