Delayed Cortical Impairment following Lipopolysaccharide Exposure in Preterm Fetal Sheep

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Objective: Preterm infants exhibit chronic deficits in white matter (WM) and cortical maturation. Although fetal infection/inflammation may contribute to WM pathology, the factors contributing to cortical changes are largely unknown. We examined the effect of fetal lipopolysaccharide (LPS) exposure on WM and cortical development as assessed by magnetic resonance imaging (MRI), electroencephalography (EEG), and histopathology in fetal sheep at preterm human equivalent age.

Methods: LPS was administered to fetal sheep at 102.5 ± 0.5 days of gestation. Continuous biophysical recordings were analyzed for 10 days after LPS. At postmortem, measurement of cerebral WM and cortical tissue volumes was achieved by stereological techniques. Specific effects of LPS on MRI-assessed T1-weighted and T2-weighted images, and immunohistochemical expression of oligodendrocytes, proliferating cells, cortical NeuN-positive and Nurr1-positive neurons (subplate marker), and cell death mechanisms were examined.

Results: We observed reductions in WM (~21%; LPS, 1.19 ± 0.04 vs control, 1.51 ± 0.07 cm3; p < 0.001) and cortical (~18%; LPS, 2.34 ± 0.10 vs control, 2.85 ± 0.07 cm3; p < 0.001) volumes, associated with overt and diffuse WM injury, T1-/T2-weighted signal alterations, and reduced numbers of WM oligodendrocytes (LPS, 485 ± 31 vs control, 699 ± 69 cells/mm2; p = 0.0189) and NeuN-positive (LPS, 421 ± 71 vs control 718 ± 92 cells/mm2; p = 0.04) and Nurr1-positive (control, 2.5 ± 0.6 vs LPS, 0.6 ± 0.1 cells/mm2; p = 0.007) cortical neurons after LPS. Moreover, there was loss of the normal maturational increase in cortical EEG amplitude, which correlated with reduced cortical volumes.

Interpretation: Fetal exposure to LPS prior to myelination onset can impair both white matter and cortical development in a preclinical large animal model, supporting a role for maternal/fetal infection in the pathogenesis of preterm brain injury.

Preterm infants have high rates of injury to white matter (WM) structures of the brain, including focal lesions or more commonly a diffuse pattern of damage involving loss of oligodendrocytes.1 By contrast, there is an apparent paucity of acute damage to gray matter (GM) structures. Nevertheless, imaging studies suggest persistent reductions in WM and GM volumes later in development in preterm born infants.2–4 Reduced cortical volumes, for instance, may contribute to cognitive deficits observed in these infants.5,6

Electroencephalography (EEG) is a well-established technique for predicting neurodevelopmental outcome after perinatal asphyxia in full-term infants,7 and may also detect brain injury in preterm infants.8 Early EEG

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depression in preterm infants is associated with increased serum proinflammatory cytokines, later neonatal brain damage, and adverse neurodevelopmental outcome. However, the relative contribution of GM versus WM damage to EEG depression in preterm infants remains unclear, and although fetal inflammation in the setting of chorioamnionitis is considered causative in preterm delivery and WM injury, a role in delayed GM deficits is unknown.

Herein, we examined the effect of fetal lipopolysaccharide (LPS) exposure on WM and cortical development and function as assessed by magnetic resonance imaging (MRI), EEG, and histopathology in 0.7 gestational age equivalent to 28 to 32 weeks gestation in humans prior to the onset of cortical myelination. Our data demonstrate a role for fetal inflammation in impairment of both WM and cortical development. Cortical alterations were reflected by loss of the normal maturational increase in EEG amplitude, and involved a marked reduction in expression of cortical NeuN-positive and Nurr1-positive neurons.

Materials and Methods

Experimental Protocol

Fetuses were randomly assigned to receive intravenous (i.v.) bolus infusion of either saline vehicle (control group; n = 11) or 200ng *Escherichia coli* LPS (055:B4; Sigma-Aldrich, St Louis, MO; LPS group; n = 9) at 102.5 ± 0.5 days of gestation (term = 147 days) (Supplementary Methods). All experimentation was approved by the Animal Ethical Committee of Gothenburg, Sweden (#307-2006) and conformed to international guidelines on ethical use of animals. All efforts were made to minimize the number of animals used and their suffering. Details on surgical procedures, physiological recordings, antibody details, and MRI acquisition and scoring parameters are shown in the Supplementary Methods.

Tissue Collection

At 10 days after LPS, ewes and fetuses were killed by i.v. overdose of sodium pentobarbitone. Fetal brains were perfusion-fixed in situ with 0.9% NaCl solution then 4% paraformaldehyde in 0.1M phosphate buffer (Histofix; Histolab, Gothenburg, Sweden). Brains were postfixed in 4% paraformaldehyde until MRI.

MRI

Fixed brains were bathed with Fomblin Profiludopolyether (Aisimont USA, Thorofare, NJ) for scanning. T1-weighted and T2-weighted (W) images were acquired on a 3T Siemens (Erlangen, Germany) Trio System with a standard wrist coil. Total brain volume measurements were performed manually on T2W images using Anatomist/Brain Visa free software. WM signal abnormalities were classified into diffuse WM signal abnormalities, periventricular WM volume loss, cystic lesions, or gliotic lesions (Supplementary Fig 1) based on T1W and T2W ex vivo MRI images (see Supplementary Methods) for qualitative assessment of WM abnormalities.

Histology and Immunohistochemistry

For each animal, a series of evenly spaced paraffin-embedded coronal sections (10μm thick, section interval = 150) were collected from the level of the anterior caudoputamen to the posterior aspects of the hippocampus of the fetal brain. Between 10 and 12 brain levels were collected per brain. Sections were stained with acid fuchsin/thionin (AF/T) for morphological analysis. Adjacent sections were stained for active caspase-3, Iba-1 (microglia), Olig-2 (oligodendrocytes throughout their lineage), myelin basic protein (MBP; mature myelin), Ki67 (cell proliferation), NeuN (postmitotic neurons), and Nurr1 (subplate neurons) (see Supplementary Methods), as previously described.

Quantitative Analysis of Brain Injury

AF/T and Iba-1 stained sections were examined for gross morphological changes by light microscopy. Microglia cell activation was assessed semiquantitatively using a scoring system (see Supplementary Methods). Olig-2–positive oligodendrocytes and caspase-3–positive cells in the intragyral WM, NeuN-positive and Nurr1-positive neurons in the overlying cortex, and Ki67-positive proliferating cells in the subventricular zone (SVZ) were counted at section levels 8 to 10 (dorsal hippocampal region), the levels of greatest WM volume changes in the LPS group, by light microscopy (×40 objective) using Stereoinvestigator Software V.7 (Microbrightfield, Williston, VT). All Ki67-positive cells in the SVZ and caspase-3–positive cells in the WM were counted; at least 10 counting sites (50 × 50μm) were used for NeuN-positive and Nurr1-positive neurons in the cortex and Olig-2–positive oligodendrocytes in the WM. All cell counts were converted to density (cells/mm3) and presented as average density of the 3 levels assessed. Volumetric changes in the corpus callosum (CC), WM (excluding CC), and cerebral cortex were assessed using the Cavalieri principle on AF/T stained sections (×40 objective) using all brain levels. A schematic of the brain subdivisions is shown in Supplementary Figure 2.

Data Analysis

Within and between group differences for mean arterial pressure (MAP), fetal heart rate (FHR), EEG amplitude, EEG spectral edge (SE), and brain volumes were evaluated by analysis of variance with time or brain level as a repeated measure, followed by unpaired 2-tailed t tests when a significant overall effect was found. Differences in qualitative MRI scores between groups were evaluated by Mann-Whitney U test. Differences in postmortem data, blood parameters, and cell counts between groups were evaluated by unpaired 2-tailed t tests. Data analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Linear regression analyses were performed to determine correlations between EEG amplitude and
WM or cortical volumes and between neuronal density and cortical volumes. Statistical significance was accepted when \( p < 0.05 \). All data are presented as mean ± standard error of the mean.

Results

LPS Causes Mild Acute Transient Hypoxia but Persisting Loss of the Developmental Increase in EEG Amplitude

There is evidence that the effects of systemic LPS on the fetal brain may be mediated by LPS-induced cardiovascular changes,\(^{16}\) although in surviving fetuses, systemic LPS causes brain injury despite maintenance of cerebral oxygen transport by elevated cerebral perfusion.\(^{17,18}\) LPS exposure was associated with an acute but transient decrease in oxygen content at 6 hours (by \( \sim 29\% \)) and mild acidosis at 2 hours and 6 hours after injection (Supplementary Table 1). There was no significant change in FHR (Fig 1A) or MAP (see Fig 1B) at any time following LPS. Acute blood parameter changes were accompanied by suppression of EEG amplitude in the LPS group at 3 to 4 hours post-LPS (\( \sim 22\% \) nadir at 4 hours; \( p < 0.05 \); see Fig 1C), which then quickly resolved to control group levels. Nevertheless, over the remainder of the experiment there was a slow rise in EEG amplitude in the control group that was not observed with LPS treatment, so that EEG amplitude was less in the LPS group from 198 hours until the end of the experiment (\( p < 0.05 \)), a period when cardiovascular and blood parameters were normal. There were no changes in EEG SE at any time following LPS and no stereotypical seizures observed in raw EEG recordings (data not shown). Thus, LPS exposure was associated with loss of the normal maturational increase in EEG amplitude, which was independent of cardiovascular effects.

LPS Reduces Cortical, WM, and CC Volumes at 10 Days of Recovery

Clinical MRI studies have shown chronic reductions in cerebral cortex and WM volumes at term age in survivors of very preterm birth.\(^3\) We hypothesized that the delayed EEG changes observed following LPS would reflect altered brain development as assessed by brain volumes. Further, as scalp EEG electrodes largely measure cortical neuronal activity, and as EEG changes were previously shown to correlate with overt GM but not WM injury in equivalent aged fetal sheep,\(^9\) EEG changes should largely reflect cortical rather than underlying WM pathology. At postmortem, LPS-treated animals had reduced brain weights (\( \sim 10\% \); LPS group, 22.7 ± 0.6g vs control group, 25.2 ± 0.7g; \( t \) test, \( p = 0.01 \)) and MRI-assessed total brain tissue volumes (\( \sim 6.5\% \); LPS

![FIGURE 1: Lipopolysaccharide (LPS) causes loss of normal electroencephalographic (EEG) maturation independent of cardiovascular effects. Ten-day recovery data showing no significant fetal bradycardia (A) or hypotension (B) following LPS exposure. Response feature analysis showed that LPS was associated with a small transient reduction of EEG amplitude at 3 to 4 hours (nadir, 4 hours; LPS group, 11.2 ± 0.5µV vs control group, 14.3 ± 1.3µV; repeated measures analysis of variance [ANOVA] + t test, \( p < 0.05 \)), followed by loss of the developmental increase in EEG amplitude at 198 to 240 hours (repeated measures ANOVA + t test, \( p < 0.05 \)) suggestive of loss of normal EEG maturation (C). Due to experimental constraints, the number of animals with EEG recordings was 6 of 11 in the control group and 8 of 9 in the LPS group. There were no differences in baseline fetal heart rate (FHR), mean arterial pressure (MAP), or EEG amplitude between the groups prior to LPS bolus. *\( p < 0.05 \).]
group, 24.4 ± 1.0 cm³ vs control group, 26.1 ± 0.8 cm³; Mann-Whitney U test, p = 0.01). Cavalieri analysis of sectioned brain tissue showed significant reductions in cerebral cortex (~18%), WM (~21%), and CC (~20%) volumes in the LPS group (Fig 2A). Changes in cerebral cortex and WM volumes were greatest at more posterior levels (see Fig 2B), whereas the CC showed a widespread anterior to posterior effect (see Fig 2C). There was a positive correlation of EEG amplitude averaged over 198 to 240 hours for each animal (delayed period of reduced EEG amplitude in LPS group) with cortical volume ($r^2 = 0.59$, $p = 0.016$), but not with WM ($r^2 = 0.23$, $p = 0.413$) or CC ($r^2 = 0.22$, $p = 0.221$) volumes. Thus, loss of the normal maturational increase in EEG amplitude with LPS is reflective, at least partially, of LPS-induced changes in the cortex, but not the WM or CC.

**In Utero LPS Causes Inflammatory White Matter Injury, Oligodendrocyte Cell Loss, and Caspase-3 Activation**

We next determined whether reduced WM volumes after LPS reflected overt WM tissue damage or persistent loss of WM oligodendrocytes. Mild to extensive diffuse cerebral WM tissue rarefaction that varied from intragryal to deep periventricular WM regions was observed in 7 of 9 animals following LPS exposure (Fig 3B, E), whereas both multifocal gliotic and cystic periventricular WM lesions were present in 4 of 9 animals in the LPS group (see Fig 3C, F; Supplementary Table 2). All fetuses exposed to LPS also exhibited a generalized pattern of increased expression of activated microglia/macrophages throughout the intragryal and periventricular WM (see Fig 3K, L; Supplementary Table 2).

Areas of periventricular WM rarefaction generally exhibited reduced expression of Olig2-positive oligodendrocytes with increased activated microglia; focal gliotic periventricular WM lesions contained increased expression of Olig2-positive oligodendrocytes, increased activated microglia, and numerous caspase-3–positive cells. Compared to control animals, there was reduced myelin expression within focal gliotic periventricular WM lesions and in adjacent areas of WM rarefaction (Fig 4B, D).
the intragryral WM, LPS exposure was associated with a significant reduction in Olig2-positive cells (≈30%; LPS group, 485 ± 31 vs control group, 699 ± 69 cells/mm²; t test, p = 0.0189; see Fig 3H, I) and a significant increase in caspase-3–positive cells (≈56%; LPS group, 0.61 ± 0.08 vs control group, 0.28 ± 0.09 cells/mm²; t
with Iba-1–positive cells with a phagocytic morphology (see Fig 5C) and expression of caspase-3–positive cells (see Fig 5D). Although this pattern of cortical injury may have contributed to the reduced cortical volumes in LPS-treated animals, the reduction in cortical volume was a more global phenomenon (see Fig 2B). There was only very low sporadic expression of caspase-3–positive cells in the cortex of both control and LPS–treated animals (data not shown). Overall, these data suggest that the cortical volume reductions following LPS were unlikely to be related to gross cortical pathology or persistent cortical apoptosis.

**In Utero LPS is Associated with Reduced Expression of Cortical Neurons**

To determine whether the reduction in cortical volume following LPS exposure was due to loss of cortical neurons, we examined expression of NeuN-positive neurons in the cortex. There was a marked decrease in the density of cortical NeuN-positive cells in fetuses exposed to LPS (41%; LPS group, 421 ± 71 vs control group, 718 ± 92 cells/mm²; \( t \) test, \( p = 0.04 \), and a positive correlation between density of NeuN-positive cells and cortical volume over the entire cohort (\( r^2 = 0.41, p = 0.02 \)). We also examined expression of Nurr1 (nuclear hormone receptor 4A2), a marker for a subpopulation of subplate neurons.20 Nurr1-positive cells were expressed mainly in cortical layers V and VI in sham-operated 100-day-old
fetal sheep (Fig 6A, C). There was a marked decrease in numbers of Nurr1-positive cells in fetuses exposed to LPS (75%; LPS group, 0.6 ± 0.1 vs control group, 2.5 ± 0.6 cells/mm²; p = 0.005), and the remaining cells were almost exclusively restricted to layer VI (see Fig 6B, D). We also examined the effects of LPS on proliferating cells in the SVZ. However, there was no difference in the numbers of Ki67-positive cells in the SVZ between groups (LPS group, 225 ± 108 vs control group, 149 ± 42 cells/mm²). Thus, LPS exposure was associated with reduced numbers of cortical neurons that likely contributed, at least in part, to the reduced cortical volumes in LPS-treated animals.

Detection of LPS-Induced Pathology by MRI

Diffuse WM damage was characterized by a diffuse T₂-hyperintense signal and a diffuse T₁-hypointense signal, whereas no such changes were observed in the control group (Fig 7). Focal periventricular WM lesions were characterized as either highly hyperintense signals on T₂ associated with very dark signal on T₁, or hypointense signal on T₂ associated with hyperintense signal on T₁. Injury scores developed from MRI data revealed a significantly higher overall score in the LPS group (control group, 5.7 ± 0.6 vs LPS group, 8.8 ± 0.6; p = 0.005). Individual scores for WM signal abnormality and gliotic lesions were higher in the LPS group, whereas scores for WM volume reduction and cystic lesions only trended to be higher in the LPS group (Fig 8).

Discussion

Clinical MRI studies suggest that infants born prematurely exhibit persistent volume reductions in both WM and GM structures later in development, and reduced cortical volumes may contribute to the cognitive deficits observed in these infants. The underlying mechanisms of this encephalopathy remain unknown, but perinatal infection and inflammation are risk factors for perinatal birth and increased neonatal cerebral morbidity. Here, we established that in utero exposure to LPS, a component of gram-negative bacteria, in fetal sheep at an age prior to onset of WM myelination, resulted in reduced WM and cortical volumes after 10 days recovery. Impairment of cortical development was further supported by loss of the normal maturational increase in EEG amplitude and a marked reduction in numbers of cortical neurons.

WM Injury and Detection by MRI

In utero LPS resulted in a pattern of occasional focal gliotic and cystic periventricular WM injury, with more common intragyral and deep periventricular diffuse WM tissue rarefaction. This is comparable with neuropathological findings in preterm infants that diffuse WM injury, rather than frank cystic lesions, is the main pathology. We also observed reduced numbers of oligodendrocytes and increased caspase-3 expression in the intragyral WM following LPS, similar to previous reports that prenatal LPS results in caspase-3-dependent oligodendrocyte loss in the cerebellar WM of fetal sheep. Nevertheless, we found no obvious change in intragyral MBP expression, although in both groups MBP staining was very low relative to the periventricular WM, making assessment of myelin changes difficult. Interestingly, expression of olig2-positive oligodendrocytes was increased in focal gliotic lesions in the periventricular WM, accompanied by reduced MBP staining that extended into surrounding tissue. Neuropathological assessment of infants with periventricular leukomalacia indicated increased olig2-positive oligodendrocytes at necrotic WM foci. Furthermore, in neonatal rats, cerebral hypoxia-ischemia caused preoligodendrocyte proliferation.
with maturational arrest and failure to myelinate in gliotic lesions. Thus, oligodendrocytes in gliotic periventricular WM lesions in our study may have exhibited arrested maturation. However, as our tissue was not optimally processed for immunohistochemical expression of oligodendrocyte stage markers, we cannot provide definitive evidence.

Histopathological findings were well supported by MRI, where T1W and T2W images clearly identified areas of diffuse WM injury and focal periventricular WM lesions. Diffuse WM lesions were characterized by a T1W hypointensity and T2W hyperintensity, corresponding to histological diffuse WM tissue rarefaction. A similar, although more intense, pattern was observed in areas with focal periventricular WM lesions. These T1W and T2W changes likely result from high water content, suggesting that this MRI pattern reflects the cystic lesions observed by histopathology. By contrast, other focal WM

FIGURE 7: Effect of lipopolysaccharide (LPS) exposure on white matter (WM) assessed by magnetic resonance imaging (MRI). Ex vivo coronal T2-weighted (A–C) and T1-weighted images (G–I) are shown in a control fetus and an LPS-exposed fetus (T2-weighted, D–F; T1-weighted, J–L) in Fomblin oil, an inert MRI solution (appears black on MRI). Note the patchy and diffuse hyperintensity on T2-weighted images (asterisks, D–F) and associated hypointensity on T1-weighted images in the WM in LPS-treated animals (asterisks, J–L), likely corresponding to the diffuse WM tissue rarefaction observed histologically. Focal areas of similar, but more intense, MRI changes were observed in the periventricular WM (thick arrows, E, K). High water content in these lesions is likely responsible for these T1 and T2 changes, suggesting that these signals reflect the cystic lesions observed by histopathology. Other focal periventricular WM lesions appeared as hypointense signal on T2-weighted images and hyperintense signal on T1-weighted images (thin arrows, D, J), corresponding to microglia/macrophage-rich areas of gliosis (see Supplementary Fig 1). No such changes were observed in the control group.
lesions showed a T\textsubscript{2}W hypointensity and T\textsubscript{1}W hyperintensity, suggestive of areas of focal histological gliosis (see Supplementary Fig 1; Fig 3), similar to the punctuate lesion observed in human premature infants. Qualitative scoring of MRI-assessed WM signal changes also showed an overall increase in WM injury with LPS. However, although T\textsubscript{1}W and T\textsubscript{2}W changes in the present study resembled clinical findings, future more extensive high-field MRI findings in experimental animal models are needed that replicate what is seen in the human condition. Of note, control brains exhibited scores suggestive of slight WM abnormalities. The invasive surgical procedures may have resulted in minor WM changes, although we observed no histological evidence of WM pathology in control cases. Alternatively, this may represent overinterpretation of MRI images.

Cortical Injury and Detection by EEG

There are few studies examining the association between perinatal inflammation and EEG in preterm infants. We found that LPS-treated animals exhibited loss of the normal maturational increase in cortical EEG amplitude. Further, this reduced EEG amplitude was correlated with reduced cortical volume. These EEG and cortical volume changes likely reflect, at least in part, the marked reduction in density of cortical neurons we observed with LPS exposure. Alternatively, cortical EEG and volume changes in our study may reflect neuronal process and synaptic deficits in the cortex. We also observed reduced expression of Nurr1-positive neurons in LPS-treated animals. Expression of the orphan nuclear receptor Nurr1 identifies a subpopulation of subplate neurons, which are believed to play an important role in the establishment of corticocortical and thalamocortical projections and in cortical development. Furthermore, subplate neurons may be particularly vulnerable to injury during development, as hypoxia-ischemia in neonatal rats selectively increased death of subplate neurons. Nevertheless, the change in this population of cells in our study may reflect a more global effect of LPS on cortical neurons.

The cause of the reduced density of cortical neurons following LPS treatment is unknown. There was minimal gross cortical pathology suggestive of acute injury, as supported by studies in preterm fetal sheep showing no acute effects of LPS on cortical neurons, despite pronounced WM injury, indicating a role for delayed processes. Nevertheless, despite ongoing WM apoptosis at 10 days post-LPS, we found no change in cortical caspase-3 expression, suggesting that cortical apoptotic cell death occurred earlier or that nonapoptotic pathways were involved. Other potential mechanisms of altered cortical development include defects in birth or migration of cortical neurons. In humans and other species, the dorsal telencephalic SVZ actively generates a population of predominantly GABAergic late migrating neurons extending into at least 25 to 27 gestational weeks, and doublecortin-positive early postmitotic migrating neurons persist in human WM up to near term. Furthermore, there is evidence of loss of GABAergic neurons in the central WM in preterm infants with WM injury. Speculatively, disruption of a population of late migrating neurons with LPS exposure may cause reduced neuronal integration into the cortex. Of note, we found no effects of LPS on proliferating cells in the SVZ, which is supported by rodent studies where LPS reduced survival of newborn hippocampal neurons, but did not affect proliferation. The potential impact of inflammation on migrating neurons requires future study.

In summary, we demonstrate a relationship between intrauterine inflammation and impaired development of cortical GM and underlying WM in a preclinical large animal model. These data provide support for a role of maternal/fetal infection in the pathogenesis of preterm brain injury. Furthermore, MRI and serial EEG may be useful to indicate different WM lesions and timing of cortical changes in these infants.
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Potential Conflicts of Interest
Nothing to report.

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