Prenatal hypoxemia alters microglial morphology in fetal sheep

Kendall M. Lawrence, MD, a Patrick E. McGovern, MD, b Ali Mejaddam, MD, a Avery C. Rossidis, MD, a Heron Baumgarten, MD, a Aimee G. Kim, MD, a Judith B. Grinspan, PhD, b Daniel J. Licht, MD, b Enrico Radaelli, DVM, PhD, c Jack Rychik, MD, d William H. Peranteau, MD, e Marcus G. Davey, PhD, a Alan W. Flake, MD, a and J. William Gaynor, MD e

ABSTRACT

Objective: Neuroimmune cells, particularly microglia and astrocytes, play a critical role in neurodevelopment. Neurocognitive delays are common in children with congenital heart disease, but their etiology is poorly understood. Our objective was to determine whether prenatal hypoxemia, at levels common in congenital heart disease, induced neuroimmune activation to better understand the origins of neurobehavioral disorders in congenital heart disease.

Methods: Eight fetal sheep at gestational age 109 ± 3 days (term ~145 days) were cannulated onto a pumpless extracorporeal oxygenator via the umbilical vessels and supported in a fluid environment for 22 ± 2 days under normoxic (n = 4) or hypoxic (n = 4) conditions. Control fetuses (n = 7) were harvested at gestational age 133 ± 4 days. At necropsy, brains were stained with ionized calcium-binding adaptor molecule 1 and glial fibrillary acidic protein antibodies to quantify microglia and astrocytes, respectively, in gray and white matter in frontotemporal and cerebellar sections. Microglia were classified into 4 morphologic types based on cell shape. Data were analyzed with 1-way analysis of variance or Fisher exact test, as appropriate.

Results: Oxygen delivery was significantly reduced in hypoxic fetuses (15.6 ± 1.8 mL/kg/min vs 24.3 ± 2.3 mL/kg/min; P < .01). Rates of apoptosis were similar in hypoxic, normoxic, and intraterine control animals in all examined areas. There were also no differences between groups in area occupied by glial fibrillary acidic protein-labeled astrocytes or ionized calcium-binding adaptor molecule 1-labeled microglia in all examined areas. However, round microglia were significantly increased in hypoxic animals compared with normoxic animals (33% vs 6%; P < .01) and control animals (33% vs 11%; P < .01).

Conclusions: Prenatal hypoxemia altered microglial morphology without significant gliosis. Additional studies characterizing these mechanisms may provide insight into the origins of neurobehavioral disabilities in children with CHD.

Nearly half of all children with congenital heart disease (CHD) repaired in infancy lag behind peers in motor, language, and memory development and will be diagnosed with a language or behavioral impairment such as autism or attention deficit disorder.1-4 These cognitive and psychosocial disabilities have been shown to severely

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Address for reprints: J. William Gaynor, MD, Division of Cardiothoracic Surgery, The Children’s Hospital of Philadelphia, 3401 Civic Center Blvd, Philadelphia, PA 19104 (E-mail: Gaynor@email.chop.edu).
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Both normal and pathologic intrauterine conditions. Using insight into neurodevelopmental delays observed in CHD, neuroimmune cells has never been studied due to the quality of life for patients and their families. Despite the scope of this problem and decades of research, there is limited understanding into the etiology of neurocognitive disabilities in children with CHD.

Recently, the recognition of prenatal brain anomalies suggests that fetal hypoxemia, a consequence of impaired cardiac output and abnormal blood streaming in CHD, may alter neurodevelopment even before birth in children with CHD. Neuroimmune cells, particularly microglia and astrocytes, play a critical role in neurodevelopment by establishing neural circuits and promoting myelogenesis. Inappropriate neuroimmune activation can permanently predispose to neurocognitive disability and neuropsychiatric disorders such as those that occur with high prevalence in CHD. The result of prolonged prenatal hypoxemia on neuroimmune cells has never been studied due to the challenges of studying fetuses in utero, but could provide insight into neurodevelopmental delays observed in CHD.

Recently, an artificial womb was developed that mimics in utero physiology and permits the study of fetuses under both normal and pathologic intrauterine conditions. Using this artificial womb model, we have previously shown that prenatal hypoxemia, at levels common in CHD, impairs myelination and reduces neurogenesis. Here we utilized this model to determine whether prenatal hypoxemia induced microglial activation or glialosis to better understand the origins of neurobehavioral disorders in CHD.

**METHODS**

**Animal Surgery and Support**

Animals were treated according to protocols approved by the institutional animal care and use committee of The Children’s Hospital of Philadelphia.

Ewe anesthesia, fetal cannulation technique, and the Extra-uterine Environment for Neonatal Development (EXTEND) support device have been described previously in detail. In brief, fetal sheep at gestational age (GA) 109 ± 3 days (term ~145 days) were exteriorized via laparotomy and hysterotomy from time-dated ewes. Fetuses were then cannulated via their umbilical vessels and attached to a pumppless low resistance oxygenator circuit. They were then transitioned to a sterile fluid environment, known as the EXTEND system, where they were supported. At cannulation, fetuses were neurologically equivalent to a 28- to 30-week preterm infant.

Fetal oxygen delivery was regulated via adjustments in the system’s sweep gas, which was a blended mixture of nitrogen, air, and oxygen. This gas mixture permitted us to maintain fetuses under normoxic (20-25 mL/kg/min), or physiologic conditions, as well hypoxic (14-16 mL/kg/min), or pathologic conditions. Levels of intrauterine hypoxia reflect those observed in severe congenital cardiac lesions such as transposition of the great arteries. Hypoxic conditions were initiated after 1 day in the EXTEND system to permit stabilization postcannulation. Throughout the study period, fetal oxygen delivery was continuously measured and recorded (LabChart 5; ADInstruments Inc, Colorado Springs, Colo) via measurement of weight-based umbilical blood flow (HXL Tubing Flowsensor; Transonic Systems Inc, Ithaca, NY), post-membrane oxygen saturation, and hematocrit concentration (M2-Sensor; Spectrum Medical, Gloucester, United Kingdom). When oxygen delivery fell outside target ranges, this triggered an adjustment in the oxygen tension of the sweep gas until the desired range was achieved.

All EXTEND system animals received continuous total parenteral nutrition and lipids (intra-lipid 20%; 0.01-0.02 g/kg/d) to mimic sheep requirements in utero. Serum chemistries were analyzed twice daily (i-STAT system; Abbott Point of Care Inc, Princeton, NJ). Dextrose and trophamine content were titrated to target a blood glucose of 20 to 30 mg/dL and blood urea nitrogen of 30 mg/dL.

At GA 132 ± 3 days, when brain maturity is equivalent to a term human, EXTEND system animals were humanely killed (pentoobarbital sodium 117 mg/kg and phenytoin sodium 15 mg/kg). Immediately following, brains were perfusion fixed with 10% formalin at 55 cm to give a hydrostatic pressure equal to physiologic mean arterial pressure (40 mm Hg). An additional 7 fetuses were delivered via caesarian-section from time-dated ewes at GA of 133 ± 4 days and humanely killed to serve as tissue controls. Their brains were perfusion fixed in identical manner described for EXTEND system fetuses.

**Histopathologic Analysis**

Cerebrum and cerebellum were removed from each skull. Using a brain matrix (Ted Pella Inc, Redding, Calif), coronal slices (4 mm) of the forebrain and cerebellum were made and then paraffin embedded. Paraffin sections 5-μm thick were stained for hematoxylin and eosin. These and all additional slides were reviewed by a Board-certified veterinary neuropathologist to assess presence, nature, and severity of histopathologic changes.

**Immunohistochemistry**

Equivalent sections from the frontal and temporal lobes were reacted with antibodies. Astroglia were visualized with rabbit glial fibrillary acidic protein (GFAP) antiserum (1:400, Z-0334; DAKO, Carpinteria, Calif). Microglia were visualized with a rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) antibody (1:1000) (158846; ABCam, Cambridge, Mass). Apoptotic cells were visualized with rabbit caspase antibody (1:100) (ab4051; ABCam). To visualize primary antibodies, sections were incubated overnight with antirabbit secondary antibodies (1:500) (ThermoFisher, Waltham, Mass) and developed with horseradish peroxidase (SK-410; Vector Laboratories, Burlingame, Calif).

Stained slides were scanned at 40× magnification and evaluated using Aperio Imagescope version 12.5 (Leica Biosystems, Buffalo Grove, Ill). Quantitative and qualitative analyses were performed on highly homologous and anatomically matched sections to avoid bias. Specific neuroanatomic landmarks, including the anterior horn of the lateral ventricle, hippocampus, and inferior cerebellar peduncle, were used to select frontal.

**Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CHD</td>
<td>Congenital heart disease</td>
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<tr>
<td>EXTEND</td>
<td>Extra-uterine Environment for Neonatal Development</td>
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<td>GA</td>
<td>Gestational age</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>Iba-1</td>
<td>Ionized calcium-binding adaptor molecule 1</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
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lobe, temporal lobe, and cerebellar sections, respectively. Periventricular white matter boundaries were defined by a horizontal line drawn tangent to the lateral ventricle at the head of the caudate nucleus, connecting to the fundi of adjacent sulci, and the white matter boundaries at those fundi. Gray matter was evaluated adjacent to these boundaries. Quantitative measures described below reflect these regions of interest (ROIs). To address potential bias with this 2-dimensional approach, ROIs were analyzed in at least 4 fields at 5× magnification (16 mm²) for each animal, and the mean values compared between groups to avoid poorly representative sampling windows.

Quantification of Apoptotic Cells
For detection of apoptotic cells, caspase-3 positive cells were manually counted in ROIs. In addition to cortical ROIs, cerebellar white and gray matter was also analyzed for caspase-positive cells.

Quantification of Iba-1 and GFAP Stained Area Fraction
Area fraction of Iba-1-positive microglia and astrocyte-positive GFAP were calculated within each ROI using a calibrated color deconvolution algorithm (Aperio Technologies, Buffalo Grove, Ill). The areas of positive staining were subtracted from the total areas of the high-powered fields studied and expressed as a percentage.

Qualitative Microglial Assessment
Iba-1-labeled cells were classified into 4 morphologic types based on their cell shape and configuration of their cytoplasmic processes. The 4 subtypes were round microglia, microglia with stout processes; microglia with thick, long processes; and microglia with thinner, more ramified processes (Figure 1). Cells were only classified if their entire, rounded cell body was present and stain appeared uniformly dark throughout the cell to avoid counting cell fragments. Ten high-powered fields were analyzed per animal in the frontal lobe periventricular white matter ROI previously described by 1 blinded reviewer. Because quantity of Iba-1-labeled cells varied between animals, microglial subtypes were compared as percentages of the total number of classified microglia.

Statistical Analysis
All analyses and graphic depictions of data were performed in GraphPad Prism V6 (GraphPad Software, San Diego, Calif). Hemodynamic parameters (ie, mean arterial pressure and circuit flow) and oxygen delivery were averaged across 12 hours and analyzed using a mixed linear effects model to determine whether a significant difference existed between normoxic and hypoxic groups over time. Histologic quantifications were analyzed using 1-way analysis of variance or Kruskall-Wallis tests, as appropriate. When significance differences existed, multiple comparisons were performed with posttest Tukey or Dunn analyses. Microglial morphologic differences were analyzed using Fisher exact tests for the phenotypic outcome of interest. All data are presented as mean ± standard deviation.

RESULTS
Animal Groups
Four cannulated animals were supported under normoxic conditions and compared with 4 animals supported under hypoxic conditions for similar durations (23 ± 3 days vs

![Figure 1](image-url)  
**Figure 1.** Representative microglial phenotypes are shown here. Microglia were classified based on their cell shape and configuration of their cytoplasmic processes. A, Round or ameoboid (lacking all cytoplasmic processes). B, Stout (microglia are round in shape but bear pseudopodia). C, Thick (rounded microglia that contain thick, flattened processes). D, Thin (microglia contain abundant, thin ramified processes).
22 ± 1 days; P = .7). Throughout the study periods, normoxic and hypoxic animals had significantly different pre-oxygenator (arterial) saturation (31% ± 6% vs 12% ± 3%; P < .01), postoxygenator (venous) saturation (60% ± 10% vs 27% ± 11%; P < .01) and oxygen delivery (24.3 ± 2.3 mL/mg/min vs 15.6 ± 1.8 mL/kg/min; P < .01). Oxygen delivery differed between normoxic and hypoxic groups despite similar umbilical blood flow (191 ± 35 mL/kg/min vs 174 ± 60 mL/kg/min; P = .7), because of significantly reduced venous oxygen tension in the hypoxic group (40 ± 10 mm Hg vs 22 ± 6 mm Hg; P = .02). Carbon dioxide levels did not differ between normoxic and hypoxic groups (39.1 ± 0.2 mm Hg vs 39.5 ± 2.5 mm Hg; P = .8). Nutrition parameters, including lipid (1.9 ± 0.3 kcal/kg/d vs 2.5 ± 1.0 kcal/kg/d; P = .3), dextrose (38.5 ± 4.3 kcal/kg/d vs 42.2 ± 4.3 kcal/kg/d; P = .3) and trophamine (25.2 ± 4.6 kcal/kg/d vs 24.4 ± 3.7 kcal/kg/d) delivery also did not differ between normoxic and hypoxic animals. Weight at cannulation (1.95 ± 0.16 kg vs 1.82 ± 0.36 kg; P = .54) and necropsy (2.91 ± 0.43 kg vs 2.59 ± 0.83 kg; P = .67) did not differ between normoxic and hypoxic animals.

Hemodynamic and caloric delivery parameters in both normoxic and hypoxic groups were similar to reported reference values for in utero sheep fetuses.19,20 Oxygen delivery in normoxic animals was similar to reported reference values for healthy in utero sheep and human fetuses,18 and oxygen delivery in hypoxic animals was similar to reported values in human fetuses with severe intrauterine growth restriction or CHD.7,8

**Gross Injury**

Cortical and cerebellar white and gray matter were qualitatively assessed for severity of brain injury by a veterinary neuropathologist. One punctate area of focal infarction was identified in the frontal lobe white matter of 1 hypoxic animal. No other infarcted areas were identified in any examined brain region of normoxic, control, or hypoxic animals. No necrotic or cystic lesions, characterized by clusters of pyknotic nuclei with reactive macrophages of astrocytes, were identified in any examined brain region of normoxic, control, or hypoxic animals. Cell counting confirmed this qualitative assessment and demonstrated similar levels of caspase-3 positive cells in all brain regions examined (Table 1).

**Area Iba-1 and GFAP Labeled Fraction**

There were no inflammatory lesions, characterized by clusters of astrocytes or microglia, identified in any brain region examined in normoxic, control or hypoxic animals. Total area occupied by GFAP-labeled astrocytes and Iba-1-labeled microglia was similar for all ROIs examined (Table 2).

**TABLE 1. Results of cell counting to determine caspase-3 positive cells in brain regions examined**

<table>
<thead>
<tr>
<th>Brain region examined</th>
<th>Apoptotic cell count</th>
<th>Control (n = 7)</th>
<th>Normoxic (n = 4)</th>
<th>Hypoxic (n = 4)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White matter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>0.8 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>0.8 ± 0.5</td>
<td>.87</td>
<td></td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>1.8 ± 0.7</td>
<td>1.3 ± 0.5</td>
<td>1.3 ± 0.8</td>
<td>.76</td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.3 ± 1.1</td>
<td>2.5 ± 1.0</td>
<td>2 ± 1.4</td>
<td>.96</td>
<td></td>
</tr>
<tr>
<td>Grey matter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>0.8 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>.45</td>
<td></td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>1.8 ± 0.7</td>
<td>0.5 ± 0.3</td>
<td>1.3 ± 0.8</td>
<td>.44</td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>.79</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>.76</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation.

**Microglial Morphology**

Figure 2 shows the distribution of microglial subtypes among groups in frontal lobe white matter. In the hypoxic animals, there was a significant increase in the round/amoeboid microglial subtype compared with normoxic (33% vs 6%; P < .01) and control animals (33% vs 11%; P < .01), which could reflect increased microglial activation or immaturity.24,25 In contrast, normoxic and control animals had a similar distribution of round/amoeboid type microglia (6% vs 11%; P = .14).

**DISCUSSION**

Using an artificial womb, we show that prenatal hypoxemia, at levels common in CHD, led to significant changes in microglial morphology. We found that these morphologic changes occurred without cell death or significant changes in the density of microglia or astrocytes. Because microglia are important regulators of neurodevelopment and determinants of postnatal neurobehavior,26 these findings may

**TABLE 2. Area density of glial fibrillary acidic protein (GFAP)-labeled astrocytes and ionized calcium-binding adaptor molecule 1 (Iba-1)–labeled microglia in brain regions examined**

<table>
<thead>
<tr>
<th>Brain region examined/ stained</th>
<th>Control (n = 7)</th>
<th>Normoxic (n = 4)</th>
<th>Hypoxic (n = 4)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White matter (% Iba-1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>7 ± 4</td>
<td>9 ± 3</td>
<td>8 ± 3</td>
<td>.64</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>7 ± 4</td>
<td>6 ± 1</td>
<td>7 ± 2</td>
<td>.83</td>
</tr>
<tr>
<td>White matter (% GFAP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>25 ± 8</td>
<td>23 ± 9</td>
<td>22 ± 7</td>
<td>.75</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>21 ± 7</td>
<td>30 ± 5</td>
<td>23 ± 9</td>
<td>.11</td>
</tr>
<tr>
<td>Grey matter (% Iba-1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
<td>4 ± 3</td>
<td>.29</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
<td>.19</td>
</tr>
<tr>
<td>Grey matter (% GFAP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>8 ± 4</td>
<td>11 ± 3</td>
<td>7 ± 2</td>
<td>.39</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>7 ± 3</td>
<td>7 ± 2</td>
<td>6 ± 2</td>
<td>.67</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation. Iba-1, Ionized calcium-binding adaptor molecule 1; GFAP, glial fibrillary acidic protein.
provide new insights into the etiology of neurocognitive disorders common to CHD.

Microglia play a critical role in neurodevelopment during fetal life by establishing neuronal circuits\(^9,10\) and promoting myelogenesis.\(^11,12\) Mature microglia are highly ramified cells with a multitude of mobile processes that continuously survey and respond to the parenchymal environment to maintain homeostasis. Under physiologic conditions, these cells prune neuronal circuits and secrete stimulating factors for oligodendrocyte differentiation, functions that are essential for the development of language, memory, and behavior.\(^27,28\) Prenatal stress has been associated with inappropriate microglial activation and delayed microglial maturation.\(^24,25\) Histologically, these alterations are identified by the presence of a round or stout microglial phenotype with loss of ramified cytoplasmic processes.\(^13,25,29\) In rodent models, prenatal stressors and subsequent microglial changes have downstream effects on axonal growth, oligodendrocyte differentiation, and myelogenesis that manifests as memory and behavioral disabilities into adulthood.\(^9,13\)

Structural changes in microglia have not previously been described in children or fetuses with CHD, but similar reductions in myelination and synaptogenesis have been identified on pre- and postnatal magnetic resonance imaging studies.\(^30,31\) Previously in this same cohort of animals, we also showed histologically that prenatal hypoxemia was associated with impaired neurogenesis and myelination.\(^15\) Here for the first time we demonstrate that prenatal hypoxemia alters microglial morphology. We speculate that altered structure could be associated with impaired microglial function and contribute to the pathogenesis of our previously observed reductions in myelin and neurons. However, additional studies need to be done to test these hypotheses.

Notably absent in this study was the presence of inflammatory or necrotic lesions. In prior autopsy series in infants with CHD and postnatal cardiopulmonary bypass animal models, global inflammatory changes and frequent, necrotic, and gliotic foci were identified.\(^32-35\) Here we found no difference in the density of inflammatory cells or apoptotic cells between in utero and normoxic controls compared with hypoxic animals. These data suggest that the presence of diffuse gliosis and necrosis is more likely the result of postnatal, rather than prenatal, injury. As such, it implies that there may be modifiable postnatal factors to prevent ongoing neurologic injury in these neonates. Unfortunately, because our assessment was limited to the prenatal period, this and other questions pertaining to postnatal implications remain speculative.

**Limitations**

There were a number of additional limitations to this study. Chiefly, we did not assess microglial function, so the implications of observed changes in microglial morphology remain speculative. Future studies have been designed to profile transcriptional changes in microglia and their secreted factors to better elucidate the relationship between altered microglia and other previously observed changes in neurodevelopment.

**FIGURE 2.** Microglia were classified according to cell shape and configuration of cytoplasmic processes. Panels show a predominance of thick and thin ionized calcium-binding adaptor molecule 1-labeled microglia in the white matter. A, Intrauterine controls. B, normoxic animals. C, Hypoxic animals. In hypoxic animals, the round and stout microglial phenotype predominates. Scale bar main panels, 100 \(\mu m\); inset panels 20 \(\mu m\). Dot plots below each panel show the distribution of microglial phenotypes in 10 high powered fields (hpf). The middle horizontal line represents the median.
alterations in neurodevelopment. Next, the duration of hypoxia exposure was limited to 22 ± 2 days at midgestation in these studies due to the technical limitations of cannulating early gestation fetuses and supporting near-term fetuses within the EXTEND apparatus. In contrast, a fetus with CHD is hypoxic throughout gestation. Although we supported fetuses at the point in gestation with the most brain vulnerability, it is possible that our observed histopathologic changes underestimate the effects of prenatal hypoxia due to the relatively shortened exposure. We have recently designed new cannula to enable more prolonged studies to closer mimic CHD conditions. Finally, due to the tremendous resource use required for these long-term studies, sample size was small for each group. It is possible that we failed to identify important differences among groups, or mistakenly identified differences when they did not exist. Encouragingly, with the exception of microglial morphology, density of inflammatory markers and apoptotic cells were strikingly conserved across all groups.

CONCLUSIONS
Despite these limitations, this study still provides novel insights into the mechanisms underlying impaired brain development in fetuses with CHD. Here for the first time we show that prenatal hypoxia alters microglial morphology without significant gliosis or necrosis. This suggests that neuroimmune activation may contribute to the neuropathology observed in children with CHD that could have important implications for future fetus-based therapies. Future studies are needed to better understand the interplay between these observed microglial changes and previously identified alterations in myelination and neurogenesis.

Webcast
You can watch a Webcast of this AATS meeting presentation by going to: https://aats.blob.core.windows.net/media/19%20AM/Saturday_May4/Mini%20Theatre/Mini%20Theatre/MT%20%2323%20%20C.%20%20Walton%20Lillehei%20Competition/MT1_4.mp4.

Conflict of Interest Statement
Authors have nothing to disclose with regard to commercial support.

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References

**Key Words:** congenital heart disease, neurodevelopment, microglia

**Discussion**

**Dr Pirooz Eghtesady (St Louis, Mo).** That was a nice presentation, and I know these are very expensive and labor-intensive preps. So, well done, and this Extra-uterine Environment for Neonatal Development system model is really cool, very elegant. I have a couple of comments and just a few questions, and the first comment, I think obviously, although the current political climate would suggest otherwise, that sheep brain and human brain are quite different with significant sulci and all the other maturational changes, and I appreciate the opportunity to go back and learn some of this stuff. The maturational process for myelination is much more rapid in sheep.

So related to that, how do these differences translate to the clinical setting that we would see in humans?

**Dr Kendall M. Lawrence (Philadelphia, Pa).** Fetal sheep are an excellent animal model for the study of neurodevelopment because they share with humans a similar susceptibility to white matter injury in terms of histopathologic and anatomic features. This is despite the relatively more rapid pattern of myelination seen in sheep that you reference. Multiple prior works have demonstrated that despite this difference, hypoxic injury in midgestation fetal sheep replicates that seen in humans. In our study, we induced hypoxia at gestational age ~110 days (0.7 gestation), at which time oligodendrocyte development is roughly equivalent to a 28-30 week preterm infant. The period of highest risk for white matter injury in humans is 23 to 32 weeks postconceptual age, which overlaps with our cannulation and period of support. Ideally we would extend the duration of extra-corpooreal support to more closely mimic the relatively more prolonged period of hypoxemia seen by fetuses with congenital heart disease (CHD), but there are technical limitations to cannulating very small sheep fetuses that we are actively working on.

**Dr Eghtesady.** My understanding from what you are saying is the changes in morphology suggest that you have more of an image for a brain then in the hypoxic environment? Is that a correct understanding?

**Dr Lawrence.** Yes, sir, that’s correct.

**Dr Eghtesady.** In your model it is a total-body hypoxia, which is a different, obviously, transposition where you have, uniquely actually, that the blood going to the brain and the heart are hypoxic as opposed to sort of total systemic, because obviously that can affect all systems. Do you think that maybe mitigates, or you would have to think of an alternative way, if that’s possible, to answer the question that you have selected?

**Dr Lawrence.** This is a limitation of the model. Ideally we would isolate hypoxic conditions to the brain to more closely mimic CHD, but we haven’t been able to accomplish that. Systemic hypoxemia affects other organ systems—as you point out. With the heart, we observed that hypoxemia affected cardiomyocyte growth. We performed daily echocardiograms on cannulated animals and found that these histologic changes did not significantly influence cardiac output or, presumably, cerebral blood flow. Given our preserved cardiac function, I don’t suspect that myocardial ischemia had an additive effect in this model.

**Dr Eghtesady.** I’m amazed you don’t have more injury.**

**Dr Lawrence.** So are we.

**Dr Eghtesady.** People have done studies with maternal hypoxia, and they saw a lot of damage and injury, and you didn’t see that. Why do you think that is?

**Dr Lawrence.** Correct, many other groups have published on intrauterine hypoxia and its effects on...
neurodevelopment in fetal sheep. Broadly, those prior models have been used to study acute hypoxic injuries such as temporary cord or carotid artery occlusion or chronic hypoxic injury such as carunclectomy, placental embolization, or maternal hypoxemia. Acute studies, such as some of those published by Dr Back, have replicated white matter injury seen in premature infants, but those models do not mimic the chronic hypoxic in utero conditions of fetuses with CHD. Models of chronic hypoxia more closely replicate conditions of CHD fetuses, but typically also have an influence on fetal substrate delivery, induce fetal hemodynamic instability, or induce a maternal stress response, all of which make it impossible to distinguish the effects of hypoxia from these other confounding factors. Our model is the first to independently study the effects of chronic hypoxia while controlling for other confounding factors such as fetal caloric delivery or maternal stress response. We have also previously shown that animals adapt over time to the chronic hypoxemic conditions with rises in peripheral angiogenic transcription factors and neovascularization of vital structures, such as the heart and brain. These adaptive responses likely limit injury over time. Dr Eghtesady. Excellent. Have you measured the cerebral blood flow or done other imaging like magnetic resonance, or are you guys contemplating that to see whether the hypoxia is the primary or if there is some other associated change that may be happening? Dr Lawrence. Unfortunately the umbilical cannulas are not compatible with magnetic resonance imaging, so we have not been able to measure cerebral blood flow that way. Other, more invasive, measurements of cerebral blood flow have been poorly tolerated by the animals. We have used ultrasound Doppler to calculate middle cerebral artery-resistive indices as a noninvasive surrogate for cerebral blood flow. Using this, we have seen that normal cerebral blood flow is maintained in extra-uterine environment for neonatal development protocol animals. We are working on using contrast-enhanced ultrasound to more precisely quantify cerebral perfusion under normoxic and hypoxic conditions.