Neuropathology as a consequence of neonatal ventilation in premature growth-restricted lambs

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INTRODUCTION

Fetal growth restriction (FGR) describes the pregnancy condition in which a fetus does not grow to its genetic potential, affecting up to 9% of all pregnancies (21). FGR is associated with stillbirth, and in survivors is strongly linked to prematurity, neonatal mortality, and morbidities (4). Past infancy, FGR is acknowledged as a significant contributor toward deficits in neurological, cardiovascular, and metabolic functions (8, 53).

Placental insufficiency is the principal cause of FGR resulting in progressive reduction in transfer of oxygen and nutrients to the developing fetus (21). Chronic hypoxia induces redistribution of fetal cardiac output resulting in body growth restriction with “brain sparing” in an attempt to ensure adequate oxygenation of essential organs (particularly brain and heart), but brain sparing is not totally neuroprotective (18, 35). FGR is associated with specific impairments in brain structure and function, where neurodevelopmental deficits depend on the fetal age at onset of poor placental function (early or late) detected by ultrasound imaging, severity of growth restriction, and gestation at birth (35). Early onset FGR, generally diagnosed in midpregnancy, is more likely to be linked with severe placental dysfunction and a greater degree of growth restriction (20). Brain development is also profoundly compromised in early onset FGR, as demonstrated in both human imaging studies and experimental animal studies (2, 13). Assessment of the neuropathology associated with early onset FGR is often confounded by the strong link between the presence of early placental dysfunction and FGR, with preterm birth (22). When FGR infants are delivered prematurely, they are likely to require neonatal intensive care, including resuscitation at birth and invasive mechanical ventilator support over the first days of life. Mechanical ventilation, particularly when poorly controlled, is associated with inflammation and lung and brain injury in appropriately grown preterm infants (12). The onset of ventilation increases early biomarkers of neuropathology in late-onset FGR lambs (1), but the interactions between chronic in utero compromise arising from early onset placental dysfunction and neonatal ventilation on the brain have not been well studied.

In this study we examined the short-term (24 h) effects of ventilation in preterm lambs with early onset FGR. There is an association between intensity and duration of mechanical ventilation and brain injury, and we hypothesized that mechanical ventilation in the neonatal period may exacerbate neuropathology associated with early onset FGR. To test this hypothesis, we used our early onset (0.6 gestation) placental insufficiency and FGR sheep model (2), with lambs delivered preterm and

<table>
<thead>
<tr>
<th>Neuronal Control</th>
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<td>Beth J. Allison, Amy E. Sutherland, Ilias Nitsos, Graeme R. Polglase, Suzanne L. Miller</td>
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ventilated using standard clinical care conditions for 24 h. We then examined the effects of neonatal ventilation on brain injury in the premature growth-restricted lamb using established physiological, histological, biochemical, and tissue immunohistochemistry methods. This would assist in designing targeted therapies for brain injury in vulnerable infants.

**METHODS**

**Ethics Approval**

Experiments complied with the National Health and Medical Research Council of Australia guidelines for the care and use of animals for scientific purposes and were approved by Monash Medical Centre Animal Ethics Committee A.

**Surgery to Induce FGR**

Single umbilical artery ligation (SUAL) was the experimental procedure used to induce FGR, as reported by us previously (38, 48). At 88 days gestation (term is 150 days), pregnant twin-bearing Border-Leicester Merino crossbred ewes underwent sterile surgery induced with sodium thiopentone (Pentothal; Bomac Laboratories, Auckland, New Zealand) and maintained under 1–2.5% isoflurane (Isoflo; Abbott Australasia, Botany, NSW, Australia). The first fetus exposed from the uterus was assigned as the control fetus [appropriate for gestational age (AGA)] in which the umbilical cord was manipulated (the outer sheath was opened and umbilical artery identified) but not ligated. In the second fetus, two silk sutures were tied tightly around one of the umbilical arteries (SUAL), ~1 cm apart, 3–4 cm from the fetal body. A single lumen polyethylene catheter (inner diameter: 0.5 mm; outer diameter: 1.0 mm; Critchley Electrical, Kingsgrove, NSW, Australia) was inserted into the femoral artery of each of the AGA and FGR fetuses, and catheters were exteriorized through an incision in the right flank of the ewe. A maternal jugular vein catheter was also implanted via an incision in the jugular groove of the right side of the neck. The ewe was recovered from anesthesia and surgery and provided analgesia (paracetamol suppository; Panadol; GSK).

**Fetal Monitoring**

For 3 consecutive days after surgery, antibiotics (ampicillin and Engenacin; Austrapen; CSL) were administered intravenously to the fetus to measure carotid blood flow as an index of cerebral blood flow (CBF). The umbilical cord was clamped and cut, and the lamb was delivered. The lamb was dried, weighed, and transferred to an incubator ventilated using standard clinical care conditions for 24 h. We then examined the effects of neonatal ventilation on brain injury in the premature growth-restricted lamb using a small sensor, which was placed over the head (the frontoparietal brain region) and covered with a lightproof (aluminum foil) dressing. Cerebral oxygenation was expressed as tissue oxygenation index (%) at 0.5 Hz. Cerebral oxygen extraction was then calculated using CBF, $\text{SP}_\text{O}_2$, and tissue oxygenation index as previously described (40).

Ventilation of FGR and AGA lambs (vent FGR and vent AGA) was initiated using assisted control ventilation (Babylog 8000 plus; Dräger, Lübeck, Germany) with an initial peak inspiratory pressure of 30 cmH₂O and positive end-expiratory pressure of 5 cmH₂O for the first 10 min. The inspired oxygen fraction ($\text{Fi}_\text{O}_2$) commenced at 0.3 but was adjusted to maintain $\text{SP}_\text{O}_2$ between 85 and 95% after initial resuscitation. All lambs received prophylactic surfactant (100 mg/kg; Curosurf, Chiesi Pharma, Italy) via the endotracheal tube at 10 min after birth. Lambs were subsequently ventilated for 24 h using volume guarantee mode with a tidal volume (VT) set at 5–7 ml/kg. The settings for lamb ventilation were based on previous studies of preterm lambs from our group, which are known not to result in significant lung injury (10, 46). Throughout ventilation, rectal temperature of the lamb was monitored and maintained within normal range (38.5–39.5°C), and lambs were kept lightly sedated by continuous infusion of Alfaxan (2–4 mg·kg⁻¹·min⁻¹; Jurox, Rutherford, NSW, Australia) via the umbilical vein catheter. Lamb well-being was assessed by regular arterial blood gas measurements via samples collected from the umbilical arterial catheter. At the completion of the experiment, lambs were humanely killed by intravenous pentobarbital sodium overdose (100 mg/kg iv; Valabarb, Rutherford). All ventilation and physiological parameters were digitally acquired using Powerlab (1 kHz) and Laboratory Chart 8 software (ADInstruments, Castle Hill, Australia).

A separate cohort of animals was euthanized without ventilation (unvent AGA and FGR) at the time of delivery (at 125 days) immediately after the cord was cut.

**Brain Processing**

After euthanasia at around 24 h after delivery, cerebrospinal fluid (CSF) was collected using a 3-ml syringe and 18-gauge needle, and the brain was then removed and weighed. The left brain hemisphere was divided into four sections (anterior to posterior) and frozen for analysis. The right brain hemisphere was coronally cut into 5-mm slices/blocks and fixed in formalin for 48–72 h and then embedded in paraffin (ProSci Tech; Thuringowa, QLD, Australia) for histological and immunohistochemistry analysis.

**Histological Staining**

Hematoxylin and eosin (H&E; cat. no. HH-1NPR and EOA1-1L; Amber Scientific, WA, Australia) staining was performed to examine for the presence of gross neuropathology. Thioflavin T (cat. no. T3516-25G; Sigma, St. Louis, MO) staining was performed to assess for the deposition of amyloid within the brain. After serial hydrations, sections were immersed in 1% thioflavin for 30–60 min, dehydrated, coverslipped, and assessed under fluorescent microscopy. Congo red stain was also used to confirm the presence of amyloid presence in tissue sections. With this technique, amyloid deposits stain red and cell nuclei blue. Briefly, sections were deparaffinized, rehydrated, and stained in Congo red solution for 15–20 min at room temperature. After being rinsed in distilled water, the sections were quickly differentiated in alkaline alcohol solution and counterstained with Gill’s hematoxylin for 30 s. After being rinsed in tap water for 2 min, the sections were dehydrated, cleared, and coverslipped.
Molecular Assessment and Cytokine Assays

Concentrations of intercellular adhesion molecule, vascular cell adhesion molecule, interleukin (IL)-3, IL-6, neuron-specific enolase, decorin, interferon-γ, IL-17A, IL-21, IL-8, IP-10, monokine induced by gamma, secreted frizzled-related protein, tumor necrosis factor-α, and vascular endothelial growth factor A were assessed in lamb serum and CSF samples using a human 5-plex and an ovine 10-plex antibody array following the manufacturer’s instructions (Cruz Biolab, Scoresby, VIC, Australia). Ovine brain-derived neurotrophic factor, nerve growth factor, and amyloid precursor protein (26) ELISAs were also conducted on homogenized brain tissue.

Immunohistochemistry of Brain Tissue Sections

Single label immunohistochemistry. Immunohistochemistry (IHC) utilizing selective antibodies for imaging-specific antigens in brain tissue was conducted. Cellular apoptosis was assessed using activated caspase-3 (cat. no. AF835; R&D Systems, Minneapolis, MN), astrocytes were assessed using glial fibrillary acidic protein (GFAP; cat. no. G3893; Sigma-Aldrich), cerebral inflammatory cells were evaluated using ionized calcium-binding adapter molecule 1 (Iba-1; cat. no. 019-19741; Wako Pure Chemical Industries, Osaka, Japan), blood-brain barrier (BB) integrity was assessed by staining with albumin (cat. no. S4265-2ML; Sigma-Aldrich), and oxidative stress was assessed using 4-hydroxynonenal (4HNE, 100 μM; cat. no. G3893; Sigma-Aldrich), cerebral inflammatory cells were evaluated using glial fibrillary acidic protein (GFAP; cat. no. G3893; Sigma-Aldrich), cerebral inflammatory cells were evaluated using glial fibrillary acidic protein (GFAP; cat. no. G3893; Sigma-Aldrich)

RESULTS

Quantitative Analysis of Brain Injury

Sections were viewed at a magnification of ×400 using light microscopy (Olympus BX-41) and examined in a blinded fashion by two investigators (A. Malhotra and M. Castillo-Melendez). Immunoreactive cell counts and/or density of stain were assessed in three fields of view within regions of interest on two slides per animal to give six fields of view per region per animal, for which an average was then calculated. Manual counts of GFAP-positive astrocytes, Iba-1 (activated or amoeboid microglia), caspase-3 (cell death) and 4-HNE (oxidative stress) were undertaken. Albumin IHC, GFAP-laminin double label, and H&E staining for neuropathological features were assessed descriptively and semiquantitatively. The percentage of endfeet perivascular astrocyte coverage of blood vessels was determined using GFAP and laminin; total number of laminin-positive blood vessels was first manually counted, after which only the blood vessels showing association with astrocyte end-feet (coverage) were counted. The percentage of blood vessels in close contact with GFAP-positive astrocytes was then calculated from the total number of blood vessels counted per field of view and expressed as percent astrocyte end-feet blood vessel coverage as previously described (16).

Statistics

Data are presented as means ± SE. Statistical comparisons were carried out using GraphPad Prism (version 5.0a; GraphPad Software, San Diego, CA). Body and organ weights, cell counts, and density staining were all analyzed by two-way ANOVA and Bonferroni post hoc tests for multiple comparisons between groups. Blood gas data were analyzed by two-way repeated measures ANOVA and Tukey posttest using SigmaPlot (version 12; Systat Software, San Jose, CA). Significance was accepted at P < 0.05.

RESULTS

Baseline Characteristics

A total of 24 lambs (n = 6 per group for unvent AGA, unvent FGR, vent AGA, and vent FGR) were studied. There were five fetal losses (3 AGA and 2 FGR) and one neonatal death (FGR), and data are not included from these animals. Body weight and organ weight-to-body weight ratios are shown in Table 1. SUAL induced placental insufficiency and FGR; body weight at birth in FGR lambs was reduced by ~15%

Table 1. Body and organ weight data of lamb groups

<table>
<thead>
<tr>
<th></th>
<th>Unvent AGA</th>
<th>Unvent FGR</th>
<th>Vent AGA</th>
<th>Vent FGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>3:3</td>
<td>2:4</td>
<td>3:3</td>
<td>3:3</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>2.8 ± 0.1</td>
<td>2.4 ± 0.2*</td>
<td>3.0 ± 0.2</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>Brain weight, g</td>
<td>45.9 ± 1.7</td>
<td>45.1 ± 0.5</td>
<td>43.9 ± 0.9</td>
<td>43.9 ± 1.9</td>
</tr>
<tr>
<td>Brain/body weight, g/kg</td>
<td>16.4 ± 0.7</td>
<td>19.5 ± 1.6*</td>
<td>14.8 ± 0.7</td>
<td>17.9 ± 1.0*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>86.3 ± 9.4</td>
<td>70.4 ± 8.2</td>
<td>127.3 ± 10.7*</td>
<td>94.4 ± 12.5*</td>
</tr>
<tr>
<td>Liver/body weight, g/kg</td>
<td>30.2 ± 1.9</td>
<td>29.2 ± 2.1</td>
<td>42.1 ± 1.5*</td>
<td>37.4 ± 3.1*</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>88.7 ± 7.9</td>
<td>78.0 ± 6.1</td>
<td>93.6 ± 4.6</td>
<td>80.0 ± 15.2</td>
</tr>
<tr>
<td>Lung/body weight, g/kg</td>
<td>31.4 ± 2.0</td>
<td>33.0 ± 2.0</td>
<td>31.6 ± 2.2</td>
<td>31.4 ± 4.2</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>22.3 ± 1.4</td>
<td>19.6 ± 1.4</td>
<td>26.7 ± 1.8</td>
<td>20.4 ± 2.8</td>
</tr>
<tr>
<td>Heart/body weight, g/kg</td>
<td>7.9 ± 0.1</td>
<td>8.2 ± 0.2</td>
<td>8.9 ± 0.6</td>
<td>8.1 ± 0.8</td>
</tr>
</tbody>
</table>

Data are expressed as ratio or means ± SE. AGA, appropriate for gestational age; FGR, fetal growth restriction; unvent, unventilated; vent, ventilated.
*Significant differences (two-way ANOVA) between FGR and AGA lamb group in body weights (P = 0.02) and brain-to-body weight ratios (P = 0.008) and between ventilated and unventilated lamb groups in liver-to-body weight ratios (P = 0.0002).
Table 2. Blood gas measurements in the first 24 h after birth

<table>
<thead>
<tr>
<th></th>
<th>1 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH F</td>
<td>7.30 ± 0.01</td>
<td>7.45 ± 0.02</td>
<td>7.35 ± 0.04</td>
<td>7.36 ± 0.05</td>
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<tr>
<td>Vent AGA</td>
<td>7.27 ± 0.03</td>
<td>7.47 ± 0.04</td>
<td>7.37 ± 0.05</td>
<td>7.33 ± 0.06</td>
</tr>
<tr>
<td>PaO2, mmHg</td>
<td>38.0 ± 5.9</td>
<td>47.3 ± 5.9</td>
<td>34.6 ± 3.1</td>
<td>39.0 ± 3.2</td>
</tr>
<tr>
<td>Vent AGA</td>
<td>35.3 ± 3.2</td>
<td>34.9 ± 2.0</td>
<td>38.2 ± 4.2</td>
<td>41.8 ± 3.6</td>
</tr>
<tr>
<td>HCO3⁻, mmHg</td>
<td>51.3 ± 4.4</td>
<td>39.8 ± 2.7</td>
<td>58.7 ± 7.1</td>
<td>45.6 ± 6.4</td>
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<tr>
<td>Vent FGR</td>
<td>52.2 ± 2.9</td>
<td>35.1 ± 4.3</td>
<td>49.9 ± 3.5</td>
<td>37.6 ± 9.6</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>3.5 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Vent AGA</td>
<td>4.2 ± 0.5</td>
<td>3.0 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>HCO3⁻, mmol/l</td>
<td>27.9 ± 2.0</td>
<td>27.5 ± 0.7</td>
<td>31.2 ± 1.2</td>
<td>24.3 ± 6.0</td>
</tr>
<tr>
<td>Vent AGA</td>
<td>23.6 ± 2.3</td>
<td>25.5 ± 1.5</td>
<td>27.4 ± 1.6</td>
<td>15.9 ± 0.8</td>
</tr>
<tr>
<td>Vent FGR</td>
<td>25.4 ± 3.0</td>
<td>27.5 ± 1.5</td>
<td>31.2 ± 1.2</td>
<td>24.3 ± 6.0</td>
</tr>
</tbody>
</table>

All values are displayed as means ± SE. AGA, appropriate for gestational age; FGR, fetal growth restriction; vent, ventilated. Two-way ANOVA showed significant (P < 0.05) time-related influences (T) on pH and P CO₂ and time-related and FGR-related (T/G) influence on lactate and HCO₃⁻.

as compared with AGA lambs [2.4 vs. 2.8 kg (unvent); 3.0 vs. 2.5 kg (31); P = 0.02, two-way ANOVA]. Brain-to-body weight ratios were also significantly increased in FGR lamb groups as compared with AGA animals (P = 0.008, two-way ANOVA). Ventilation caused a significant increase in liver-to-body weight ratios in both AGA and FGR lambs (P = 0.0002, two-way ANOVA).

Physiological Parameters and Ventilation Requirements

Fetal blood gas parameters following SUAL induction have previously been reported for the unvent AGA and FGR groups (2), and the fetal blood gases for the ventilated groups were comparable to previously reported data. In brief, while there were no significant differences in pH, PaCO₂, PaO₂, or SaO₂ in the first 7 days after SUAL-induced FGR onset in either AGA or FGR lambs, there was a significant difference (P < 0.05) in PaO₂ and SaO₂ on day 10 after the onset of FGR. Blood gas parameters after birth are shown in Table 2, for the vent AGA vs. vent FGR lambs. Overall, there was a significant time-related influence on pH and PaCO₂ and time and growth-related influence on lactate and HCO₃⁻ (two-way ANOVA, P < 0.05). There were no significant differences in the ventilation requirements of the FGR lambs as compared with AGA lambs throughout the experiment (Table 3).

Figure 1 summarizes the changes in cerebral tissue oxygenation index, cerebral oxygen extraction, and CBF in vent AGA and vent FGR lambs. There was a significant difference in the mean cerebral blood flow measured as carotid blood flow, wherein CBF was lower in the vent FGR lambs over the duration of the study (P = 0.01) compared with vent AGA lambs. Tissue oxygenation index also tended to be lower in the FGR lambs over the duration of the experiment and cerebral oxygen extraction higher in the FGR group (P = 0.12 and P = 0.06, respectively).

Neuropathology

We first stained all brains with H&E to assess whether there were areas of gross neuropathology associated with FGR and/or ventilation. Our baseline group of animals, the unvent AGA lambs, demonstrated normal structural integrity and white matter organization, with no evidence of red blood cell (RBC) infiltration (hemorrhage) in any brain region examined (Fig. 2. A and E, shows normal white matter within PVWM). In the vent AGA group, we observed accumulation of RBCs and inflammatory cell cuffs around some capillaries within the SCWM and PVWM (Fig. 2. C and G); however, these were confined to the capillary lumen. Unvent FGR brains showed a mild degree of infiltration of RBCs, accompanied by perivascular infiltrates of inflammatory cells within the white matter (SCWM and PVWM) consistent with microbleeds (Fig. 2. B and F). In the vent FGR group, we observed large numbers of RBCs (Fig. 2. D and H) within the vascular lumen (consistent with vascular congestion) as well as RBC infiltration into the brain parenchyma within the SCWM and PVWM, which was accompanied by a loss of the capillary wall (Fig. 2 I) and inflammatory cell infiltrates (Fig. 2. K and L). Vent FGR animals also displayed WM disorganization, hypocellularity, and cavity formation in the PVWM (Fig. 2 I) consistent with periventricular leukomalacia (Fig. 2 I).

We examined for the presence of axonal injury using two standard staining techniques. Axonal injury was not observed in any AGA brains, whether vent or unvent (Fig. 3, A–C). In contrast, axonal injury (positive for thioflavin staining) was present in four out of six unvent FGR brains and five out of six vent FGR brains (Fig. 3). This axonal injury was observed across a number of brain regions but was quite irregular in both

Table 3. Ventilation requirements in the first 24 h after birth

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<thead>
<tr>
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<th>1 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>Compliance, ml·g⁻¹·cmH₂O⁻¹</td>
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<tr>
<td>Vent AGA</td>
<td>0.016 ± 0.02</td>
<td>0.016 ± 0.02</td>
<td>0.018 ± 0.008</td>
<td>0.02 ± 0.013</td>
</tr>
<tr>
<td>Vent FGR</td>
<td>0.011 ± 0.003</td>
<td>0.016 ± 0.006</td>
<td>0.011 ± 0.001</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>Peak pressure, cmH₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vent AGA</td>
<td>16.8 ± 1.6</td>
<td>21 ± 1.6</td>
<td>21 ± 2.6</td>
<td>25 ± 3.4</td>
</tr>
<tr>
<td>Vent FGR</td>
<td>18.6 ± 6.4</td>
<td>20 ± 3.6</td>
<td>19.6 ± 3.4</td>
<td>26 ± 6.4</td>
</tr>
<tr>
<td>Tidal volume, ml/kg</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vent AGA</td>
<td>5.2 ± 0.3</td>
<td>4.9 ± 0.3</td>
<td>5.0 ± 0.3</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>Vent FGR</td>
<td>5.2 ± 0.4</td>
<td>5.0 ± 0.1</td>
<td>4.6 ± 0.3</td>
<td>4.8 ± 1.0</td>
</tr>
<tr>
<td>FIO₂ %</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vent AGA</td>
<td>40.0 ± 11.2</td>
<td>22.6 ± 1.0</td>
<td>26.0 ± 5</td>
<td>31.0 ± 10</td>
</tr>
<tr>
<td>Vent FGR</td>
<td>36.5 ± 10.5</td>
<td>22 ± 1.2</td>
<td>27.6 ± 4.0</td>
<td>32.8 ± 15</td>
</tr>
</tbody>
</table>

All values are displayed as means ± SE. No significant differences seen. AGA, appropriate for gestational age; FGR, fetal growth restriction; vent, ventilated.
size and position and predominantly within white matter regions (Fig. 3 shows staining within the SCWM). Thioflavin staining of amyloid deposits were identified by the presence of apple green fluorescence (Fig. 3, F and I), showing compact plaques with homogeneous material radiating from the center of the plaques (Fig. 3 I). We confirmed the presence of axonal injury within FGR brains using Congo red staining (Fig. 3, D, E, G, and H) under light and fluorescent microscopy, in which dense masses of axonal retraction were observed.

**Immunohistochemistry**

Neuroinflammation was assessed by the presence of astroglia (GFAP-positive staining) and activated microglia (Iba-1-positive staining). Astrocyte cell counts were similar across the unvent AGA, unvent FGR, and vent AGA groups for all brain regions examined, with the exception of a significant astrogliosis within cortical gray matter in vent AGA brains, compared with unvent AGA brains (P < 0.05; Fig. 4). The brains of vent FGR lambs demonstrated profound astrogliosis, with increased GFAP-positive cell density compared with all other groups, in the respective brain regions examined (P < 0.05) (Fig. 4). A similar finding was also seen for activated microglia within the PVWM, SCWM, and SVZ, wherein vent FGR brains showed significantly higher numbers of activated microglia compared with all other groups (Fig. 5).

During our quantification of microglia and astrocytes, we observed that the morphology of these glial cells appeared different across groups (Fig. 4A). While microglia cell counts were unchanged, microglia in unvent FGR, vent AGA, and vent FGR brains were shifted toward an activated (amoeboid) state, characterized by swollen ramified cells with a larger cell body and shorter, thick processes. In contrast, unvent AGA...
brains showed resting microglia cells with small cell bodies and many branching processes in all regions examined. Similarly, the astrocytes within the unvent FGR, and vent AGA brains were more likely to appear reactive as evidenced by cell body hypertrophy, loss of astrocyte domain, and overlapping astrocytic processes (Fig. 4). This neuropathology was accompanied by a disrupted interaction of astrocyte end-feet with cerebral blood vessels, which was only evident in FGR brains; thus we consequently studied this association using GFAP-laminin double label immunofluorescence (Fig. 6). Although it was apparent that FGR led to dissociation of the astrocyte foot processes from the blood vessels in response to ventilation in a number of FGR animals (as represented in Fig. 6), quantification of this association did not demonstrate a significant difference in the percent coverage of blood vessels overall (Fig. 6).

Disruption of the astrocyte barrier associated with compromise of the BBB was examined using albumin staining. We did not observe albumin extravasation into brain parenchyma surrounding blood vessels in any of the unvent AGA brains; however, semiquantitative analyses showed that there was BBB disruption (albumin extravasation) within the SCWM and
PVWM of the vent FGR lambs, to a greater extent than in vent AGA and unvent FGR animals (Fig. 7), suggesting BBB disruption associated with growth restriction.

Caspase-3-mediated cell death was examined (Fig. 8), and it was noted that caspase-3 staining was present within all groups and brain regions examined. Ventilation increased caspase-3-mediated cell death in the AGA group, with a significant elevation in cell counts within the PVWM (unvent AGA vs. vent AGA; \( P < 0.05 \)). The most notable increase in caspase-3-mediated apoptosis was seen in the vent FGR group, in which caspase-3 cell counts increased twofold increased in PVWM, CGM, and SVZ regions of vent FGR, compared with unvent AGA and unvent FGR (\( P < 0.05 \) for all). Furthermore, ventilation increased cellular apoptosis in vent AGA brains compared with unvent FGR within the PVWM and CGM and SVZ (\( P < 0.05 \)), indicative that ventilation per se exacerbates neuropathology in FGR offspring.

We examined cellular oxidative stress in the form of lipid peroxidation (Fig. 9) in white matter brain regions (PVWM and SCWM) of all groups, via immunohistochemistry for 4-HNE, one of the most bioactive and widely studied lipid peroxidation product. 4HNE-positive cells were significantly increased number (\( P < 0.05 \)) in the PVWM and SCWM of vent FGR lambs as compared with unvent and vent AGA lambs, and vent FGR was significantly increased above unvent FGR (\( P < 0.05 \)).

Cytokine Analysis

There was no significant change (data not shown) in any of the cytokines studied in serum or CSF in the FGR or
ventilated group as compared with unventilated controls or FGR lambs.

**DISCUSSION**

We examined short-term markers of neuropathology in response to early neonatal care and mechanical ventilation in FGR and AGA preterm lambs. Our results demonstrate that ventilation in preterm FGR lambs induces neuropathology compared with AGA lambs, with an increase in neuronophagia, oxidative stress, altered BBB structure, and cell death. This is critical information, which provides knowledge on the injurious pathways behind the increased neurological deficits seen in preterm FGR infants and provides insight for targeted strategies to improve outcomes in this vulnerable cohort of infants.

This study is the first to mimic an early onset placental insufficiency/FGR (0.6 gestation), and subsequent preterm delivery at ~0.8 gestation, with standard neonatal care and non-injurious mechanical ventilation to examine the effects of the first 24 h of life on markers of neuropathology. Early onset FGR in the human presents in the second trimester of pregnancy and is associated with a higher risk of neurological deficits including motor, cognitive, and behavior dysfunctions (13). This may be contributed by the in utero compromise to brain development, with the period from 24 to 32 wk gestation being the period at high risk for white matter damage (7). We undertook the technique to induce FGR during this vulnerable developmental period and before the onset of myelination in the periventricular white matter of the sheep brain, when preoligodendrocytes are the predominant cell type (7). The majority of infants who are diagnosed with early onset FGR will be born preterm, with the mean gestational age for delivery of this cohort of infants between 32 and 34 wk (27). We delivered our cohort of lambs at 125–127 days gestation, which is a moderate preterm age for lung maturity and late preterm, near term age with respect to brain development (6, 33). This timing in the sheep presents a neurodevelopmental stage when cortical myelination is quite advanced but the white matter remains highly vulnerable to injury. Indeed, we observed that white matter damage within FGR lamb brains that received ventilation were highly susceptible to oxidative stress, elevated inflammatory cell activation, and apoptosis-mediated cell death. The neonatal delivery and care of FGR lambs encompass all aspects of neonatal intensive care management in the preclinical environment, including thermoregulation, surfactant therapy, mechanical ventilation, fluid therapy, and antibiotics, enabling us to study the effects of standard early neonatal management on brain pathology. An interesting finding was an increase in liver-to-body weight ratio with ventilation in FGR and AGA groups, which we consider most likely to be due to the fluid therapy in this experiment and a relatively immature renal system (50).

During the 24-h period of neonatal care we noted that the FGR lambs demonstrated a reduced mean carotid blood flow (as a surrogate for cerebral blood flow) compared with AGA lambs. This is in keeping with our previous work to show that in the fetal and early newborn period, FGR lambs have a significantly lower CBF than appropriately grown lambs (36, 39). These results are at odds with clinical data obtained in the first 3 days of life indicative of elevated cerebral oxygenation in preterm small for gestational age infants using near infrared spectroscopy (17). In part, these differing observations probably reflect a degree of heterogeneity in the small for gestational age human infants, including duration and degree of fetal hypoxia, and also the techniques used to measure cerebral hemodynamics (carotid flow in sheep versus near infrared spectroscopy assessment of cortical vessels in humans). Reflecting this, clinical and animal studies have shown that there are brain region-specific changes in cerebral blood flow in growth-restricted fetuses, and cerebral hemodynamic change with advancing fetal compromise (23, 36). We would argue that the decrease in CBF observed in the this study is explained

**Fig. 7.** Representative photomicrograph of increased blood brain barrier permeability (34) seen in the cortex of a vent fetal growth restriction (FGR) lamb brain using albumin immunohistochemistry. Table shows number of animals (out of total in each group) with albumin extravasation in brain tissue. AGA, appropriate for gestational age lamb.

<table>
<thead>
<tr>
<th></th>
<th>Unvent AGA</th>
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<th>Vent AGA</th>
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<tr>
<td><strong>Albumin extravasation</strong></td>
<td>0/6</td>
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**Fig. 8.** A: representative photomicrographs of cell death using caspase-3 immunostain seen in periventricular white matter (PVWM) of unventilated (unvent) and ventilated (vent) appropriate for gestational age (AGA) and fetal growth restriction (FGR) lambs. B: significant differences (*P < 0.05) in caspase immunoreactivity in vent FGR lamb brains as compared with unvent AGA and unvent FGR; significant differences (#P < 0.05) between vent AGA compared with unvent AGA; and significant differences (*)P < 0.05) between vent FGR compared with unvent AGA lambs in respective brain regions (n = 6 each group).
by structural cerebrovascular adaptations that occur in response to chronic in utero hypoxia, with a significant decrease in vascular density in white matter brain regions in FGR lambs (15). Furthermore, we observed profound neuroinflammation within FGR brains exposed to ventilation, often accompanied by disassociation of astrocyte end-feet from blood vessels. A critical role of the astrocytes is to regulate cerebral blood flow, which they do via connections with blood vessels (24). We showed an overall decrease in tissue oxygenation within the FGR brain and increased cerebral oxygen extraction. Combined, these findings are physiologically important and indicative that the developing FGR brain is very sensitive to altered cerebral hemodynamics that arise from routine neonatal care, and these can impact on cerebral metabolism and potentially contribute to subsequent brain injury seen in this high-risk population.

There is evidence to suggest that early invasive ventilation in premature infants (who are appropriately grown) is associated with increased risk of intraventricular hemorrhage (3) and white matter injury (12, 42), particularly when the ventilation is not well controlled. It is likely that ventilation-induced brain injury in preterm infants arises due to disruption of the BBB, mediated via central inflammation and oxidative stress (11). We saw evidence of reduced BBB integrity in response to ventilation, most notable in the FGR cohort of animals. This is in agreement with our previous findings of altered BBB structure and function in FGR neonates (1, 15). Structural integrity of the BBB is critical to ensure peripheral inflammatory cells and RBCs cannot access the developing brain. Similar effects of increased BBB permeability have also been noted with ventilation of well grown preterm lambs (9), but here we show that FGR increases the vulnerability for a compromised BBB, potential RBC translocation, and subsequent intraventricular hemorrhage and white matter damage.

Neuroinflammation plays a central role in the development of brain injury in FGR infants (26, 52). Until now it has not been well defined whether the neuroinflammation present in the FGR brain was primarily antenatal in origin as a result of placental insufficiency or initiated by postnatal interventions, including ventilation, or indeed a combination of both. In preterm infants, Leviton et al. (28) proposed a two-hit model of inflammation, born small and exposed to postnatal systemic inflammation. In preterm lambs, our group has shown that ventilation increases systemic and cerebral inflammatory markers (9) and that the combination of antenatal chorioamnionitis and postnatal ventilation is associated with an exacerbation of inflammatory markers and increased brain injury (10, 43). Results from this study now further demonstrate that the two-hit model of inflammation and brain injury is appropriate for FGR offspring wherein ventilation exacerbates gliosis in FGR preterm lambs. Thus the combination of chronic antenatal hypoxia and postnatal ventilation appears to have additive adverse effects on the FGR brain, which together may contribute to long-term neurological deficits.

The contribution of microglia and astrocytes is complex, as they mediate both neurorepair and damage (5, 19, 47). In the perinatal brain, it is however increasingly well described that microglial activation is a first critical step in the progression of neuroinflammation and contributes to white matter injury (19). An upregulation of activated astrocytes and microglia in response to standard ventilation in preterm FGR infants is likely to be a contributor toward increased cellular apoptosis as observed in this study. Microglia act as a neuropathology sensor in the central nervous system and can rapidly detect subtle changes in brain tissue both in the developing and adult brain (30). In the activated form, microglia cells produce a plethora of proinflammatory cytokines and chemokines, which have been implicated in the pathogenesis of a number of pathological conditions, including cerebral palsy (25). Microglia also play an indispensable role in building the normal brain architecture and are known to be involved in myelination, phagocytosis of apoptotic neurons, axonal pruning, and the development of vascular and axonal networks (44). Given the crucial role of microglia in regulating brain development, maturation, and network connectivity, modifications to microglia cells in the developing FGR brain could alter on-going developmental processes and thereby have impact on normal brain structure. This neuroinflammatory response may well be amenable to umbilical blood cell therapy as seen in a preterm brain injury model reported previously (29).

Ventilation increased oxidative stress, as measured by 4HNE within the FGR lamb brains. This is interesting, especially in the context of similar ventilation requirements between the FGR and AGA animals. Increased expression of oxidative stress markers is seen in brain injury associated with FGR (45, 51). Our group has previously shown an increase in oxidative stress markers in serum and brain tissue of FGR lambs (38). Similarly, increased oxidative stress markers have
been seen in hypoxic and ventilation-induced brain injury models of preterm birth (14, 41). An upregulation of lipid peroxidation within the immature brain may be catastrophic for white matter development (7, 38). It is possible the FGR lamb brain has a reduced antioxidant capacity (54), which is unable to respond to another stressful state (ventilation) leading to a heightened response as seen here. In this respect it will be important to follow-up on the potential for antioxidant therapies, such as melatonin, to mitigate antenatal and postnatal contributions to neuropathology in FGR offspring (38).

We used two markers of axonal injury, thioflavin and Congo red; both of which confirm the presence of abnormal amyloid deposition occurring in response to axonal damage and local accumulation of amyloid precursor protein. Previously we have shown amyloid precursor protein deposition in term unventilated FGR lamb brains (38), and in this study we examined whether ventilation might specifically induce axonal injury in FGR or AGA lambs. Our results are indicative that some, but not all, FGR brains show evidence of axonal injury within white matter regions, but that ventilation per se did not exacerbate axonal injury. This observation also supports the two-hit model of brain injury in FGR offspring, with this underlying white matter injury and axonal damage induced during the period of chronic hypoxia in utero and additional neuroinflammatory compromise possibly initiated with neonatal ventilation.

We did not see any significant or characteristic cytokine profiles in blood, CSF or brain tissue in this study. We have previously seen some increase in IL-8, a proinflammatory cytokine in brief periods of mechanical ventilation in (late onset) FGR preterm lambs (1). This suggests a brain-specific neuroinflammatory process and further highlights the case to identify and develop organ specific biomarkers for the detection and assessment of FGR-related brain injury (32).

We acknowledge the limitations of this study. By delivering these lambs at 125 days of gestation we were able to replicate the effects of ventilation on brain development equivalent to late preterm human gestation; however, lung development is slightly more immature in the sheep at this age (equivalent to 28–30 wk gestation), such that we could not maintain the lambs past 24 h. Human infants born preterm would likely be exposed to longer periods of invasive or noninvasive ventilation and other neonatal interventions (e.g., caffeine therapy, therapy for patent ductus arteriosus), which we have not accounted for, and may separately influence brain development. All animals were exposed to antenatal glucocorticoids, which also would be likely to affect our observations within the brain (37). These findings do however give us an indication of mechanisms and mediators of early brain pathology that may be potentially modifiable in FGR infants.

**Perspectives and Significance**

Preterm growth-restricted lambs when ventilated show an increased risk of brain injury, especially in white matter brain regions, as compared with well-grown and unventilated growth-restricted lambs. Neuroinflammation and oxidative stress are upregulated and are likely to contribute to cerebrovascular compromise and apoptosis-mediated cell death. This fits in well with the two-hit hypothesis wherein chronic in utero hypoxia and a second stress of neonatal ventilation combine to result in neuropathology associated with FGR. Targeted therapies to mitigate the effects of ventilation on the growth-restricted infant brain need to be evaluated with this information in mind.

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**REFERENCES**


BRAIN INJURY IN PRETERM GROWTH RESTRICTION


