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Regular Article

Detecting brain injury in neonatal hypoxic ischemic encephalopathy: Closing the gap between experimental and clinical research

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ABSTRACT

Moderate to severe neonatal hypoxic ischemic encephalopathy remains an important cause of infant death and childhood disability. Early and accurate diagnosis of encephalopathy is difficult but critical for timely intervention. Thus, we have utilized a clinically relevant large animal model of asphyxia in-utero, followed by immediate lamb delivery, resuscitation and clinical care over the next 72 h for assessment of potential biomarkers of brain injury. In-utero asphyxia was induced in twelve near-term lambs and outcomes compared with seven controls. Asphyxia resulted in bradycardia (97 ± 12 beats/min), hypotension (12.1 ± 1 mm Hg) and metabolic acidosis (pH 6.9 ± 0.02; base-excess −13.8 ± 0.8 mmol/l). 72 h following asphyxia, cerebrospinal concentrations of malondialdehyde and S100B were elevated 2-fold and 5-fold, respectively, in asphyxic lambs compared to control lambs. Magnetic resonance spectroscopy (MRS) at 72 h showed a significant decrease in n-acetyl aspartate: choline ratio in asphyxia lambs compared to that observed at 12 h (0.56 ± 0.23 vs. 0.82 ± 0.15, respectively); lactate:choline ratio was not changed over this time. Marked neuropathology was observed in asphyxia lambs with neuronal degeneration in the hippocampus, thalamus, striatum and cortex. Astrogliosis was observed in the hippocampus and thalamus. Early blood markers of metabolic state showed limited predictive value of histological damage at 72 h. MRS outcomes at 72 h showed a modest but significant correlation with histological evidence of neuronal brain injury (lactate:N-acetyl aspartate ratio in the thalamus $r^2 = 0.2$, $p < 0.01$). MRS at 72 h was best able to detect established brain injury, but a combination of biomarkers over multiple phases of injury may be able to assess the evolution of neonatal brain injury.

Introduction

Neonatal hypoxic ischemic encephalopathy (HIE) remains an important cause of perinatal death and long-term developmental disability. Clinical trial meta-analyses demonstrate that approximately 60% of untreated (normothermic) infants will die or have long-term disability after HIE (Edwards et al., 2010). For surviving infants, there is a well-described association between HIE and diagnosis of cerebral palsy (Badawi et al., 1998). Currently, the only effective treatment to reduce adverse outcome following term HIE is hypothermia commencing within 6 h of delivery (Jacobs et al., 2013), however 47% of treated newborns are still at risk of death or serious disability (Jacobs et al., 2013). It is thus recognized that adjuvant or alternative treatments for HIE are necessary (Bennet et al., 2012; Miller et al., 2012; Robertson et al., 2012).

In response to asphyxia at term birth, brain injury and encephalopathic symptoms evolve from hours through to weeks (Gunn and Gluckman, 2007; Williams et al., 1991). This is characterized by a primary insult phase where neuronal degeneration begins. Delivery of the baby and, in association with resuscitation, leads to apparent recovery during the *latent phase*. However the *secondary phase* introduces biochemical cascades such as excitotoxicity, oxidative stress and inflammation, and subsequent neuropathology to neuronal populations in the cerebral cortex, hippocampus, thalamus, basal ganglia and to a lesser extent in white matter (Azzopardi and Edwards, 2010; Folkerth, 2005; Gunn and Bennet, 2009; Inder, 2000; Thayyil et al., 2010).

Early identification of HIE is essential for successful initiation of neuroprotective therapies. The results of hypothermia studies highlight that treatment should begin early for maximal benefit (Gunn et al., 1999). Clinically, there is an initial focus on observational parameters of encephalopathy, first described by Sarnat and Sarnat, to reflect graded

Abbreviations: aEEG, amplitude integrated electro-encephalogram; Cho, choline; DWI, diffusion-weighted imaging; GFAP, glial fibrillary acidic protein; HIE, hypoxic ischemic encephalopathy; HR, heart rate; Lac, lactate; MAP, mean arterial pressure; MDA, malondialdehyde; NAA, N-acetylaspartate; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; SaO_2, oxygen saturation; SEM, standard error of the mean.

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abnormalities in infant reflexes, behavior and tone, and incidence of seizures (Sarnat and Sarnat, 1976). More recently with the NICHD hypothermia trial for HIE, early (<6 h) neurological classification utilizing clinical and laboratory parameters showed good predictive value (78%) for death/disability, and therefore infants that would benefit from hypothermia (Ambalavanan et al., 2006). Magnetic resonance imaging (MRI) studies of the newborn brain confirm neuropathology after perinatal asphyxia and are predictive for long-term impairment (Azzopardi and Edwards, 2010; Thayyil et al., 2010). However MRI is usually performed 2–6 days or later after the sentinel insult; with later scans showing greater predictive value (Azzopardi and Edwards, 2010). Ideally, neonatal care would incorporate specific biomarkers of HIE severity and neuropathology to guide therapeutic intervention and prognosis.

The aim of this study was to cause HIE in a clinically useful large animal model in which we could correlate physiological, biochemical, radiological and histological markers of neuronal cell degeneration. This model provides endpoints selected to assess the evolution of brain injury during clinical care, to inform timing for therapeutic interventions, and to guide future neuroprotection studies.

Materials & methods

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.

Surgery and hemodynamic recording

Near-term pregnant ewes at 139–141 days gestation (term is 145–147 days) underwent sterile surgery under general anesthesia induced by sodium-thiopentone (20 mg/kg IV bolus; Pentothal, Boerhringer Ingelheim, Australia) and maintained with 1–2.5% isoflurane (Isoflow, Abott Pth) in oxygen/nitrous oxide (O_2: 2–3 L;N_2O: <1 L). The hindquarters of the fetus were exteriorized and a femoral artery cannula inserted for continuous digital recording of mean arterial pressure (MAP) and heart rate (HR; Powerlab SP, ADInstruments), and blood sampling. A femoral vein catheter was inserted. Pulse oximetry (MasimoSet Rainbow, Radical7, Masimo) was continuously monitored via a cuff placed on the shaved tail of the lamb.

Asphyxia and resuscitation

Fetal sheep were randomly allocated using an envelope assignment system to *control* or *asphyxia* groups. Asphyxia was induced via complete umbilical cord occlusion. With the head and upper body remaining in-utero in the amniotic fluid, the umbilical cord was exposed and clamped. Lambs in the control group had the cord clamped and cut immediately and were delivered and resuscitated. Lambs in the asphyxia group remained in-utero until MAP decreased to 18–20 mm Hg; shown previously in late gestation fetal sheep to induce severe asphyxia and neuropathology (Castillo-Meléndez et al., 2004). The cord was then cut and lambs delivered and resuscitated.

Australian and New Zealand Neonatal Resuscitation Council guidelines for resuscitation were followed. At delivery, all lambs were placed on an infant warmer, intubated (4.5 endotracheal tube; Portex), and dried with towels. Positive pressure ventilation (Neopuff™, Fisher & Paykel Healthcare; 30 cmH_2O positive inspiratory pressure [PIP], 5–8 cmH_2O positive end expiratory pressure [PEEP], 10 L/min room air, 30 breaths/min) was initiated. If required, lambs were administered adrenaline (1 ml) and fluid (normal saline; 20 ml/kg). As per guidelines, oxygen saturation (SaO_2) was targeted at 85–90% within 10 min. Lambs were ventilated (Babylog 8000+, Draeger; volume guarantee 5 ml/kg, PEEP 5–7 cmH_2O, 30 breaths/min) as required. Ventilation parameters were decreased and changed to continuous positive airway pressure ventilation and assisted ventilation, subsequently, ceased if the lambs began to spontaneously breath >50% of the time. Overt clinical seizures were assessed by clinical personnel (AM, MCF, FYW, EMW) as repetitive eye movements, "smacking" of the lips, neck arching, "running" leg movements, and apneic episodes, similar to human seizures as described by Volpe (Volpe, 2001). Seizures were treated with 20 mg/kg intravenous phenobarbitone (Sigma, Australia).

Maintenance

Arterial blood samples (250 μl) were collected in-utero, during asphyxia, and at 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h postnatal for assessment of pH, PaO_2, $PaCO_2$, lactate, glucose, bicarbonate and base-excess (ABL Blood Gas Analyzer, Radiometer, Denmark). HR, SaO_2, rectal temperature, and body weight were assessed hourly. When the lambs were extubated, alert, and had a strong suckle reflex, they were offered sheep milk replacer orally every 4 h (~100–150 ml; Wombaroo Food Products, Australia). Maintenance intravenous fluid (10% dextrose; 40 ml/kg/day) was commenced if lambs were unable to feed orally. Lambs were maintained for 72 h.

S100B and malondialdehyde

S100B ELISA was performed as per manufacturer's instructions (DiaSorin, Minnesota, USA) on serum (50 μl) at time 0 (in-utero), and 1, 2, 4, 8, 12, 24 and 72 h postnatal, and on CSF obtained at post-mortem. Assay sensitivity was 0.03 μg/l with a run imprecision of <10% and total imprecision of <15%.

Malondialdehyde (MDA) was measured in plasma (100 μl) at the same time-points as for S100B, using a thiobarbituric acid reacting substance assay (Miller et al., 2014). The assay sensitivity was 0.1 μmol/l, with 5.1% inter-assay and 3.1% intra-assay coefficients of variation.

Magnetic resonance imaging and spectroscopy

Brain magnetic resonance imaging and spectroscopy (MRS) were performed under sedation at 12 and 72 h after birth, using a 3 T Siemens Vario (Siemens Medical Solutions, USA) in an eight-channel knee coil (400 × 420 × 310 mm). Sedation was induced using Domitor (0.1 mg/kg iv; Pfizer Australia) a synthetic alpha-2-adrenoreceptor agonist; and reversed with the antagonist, antisedan (0.1 mg/kg; Pfizer Australia). Conventional sequences were obtained with sagittal diffusion-weighted-imaging (DWI) utilizing a slice thickness of 2 mm, 20 ms echo time, and 28 ms repetition time, T1-weighted (repetition time/echo time/excitations 1900/3.92/1), and T2-weighted (5000/90/1). MRS utilized a 270 ms echo with a 2 cm^3 voxel placed over the hippocampus, striatum and basal ganglia. MRS produced spectrographs of concentrations (calculated by computer algorithm as area under the curve) of lactate (Lac), choline (Cho), and n-acetylaspartate (NAA).

Brain pathology

Lambs were euthanased (pentobarbitone, Lethabarb Virbac Australia) immediately after the 72 h MRI. The right cerebral hemisphere was cut coronally into 5 mm slices and immersion fixed in 4% paraformaldehyde for 48 h, paraffin embedded and then coronally sliced at 10 μm. The left cerebral hemisphere was separated into anatomical locations, snap frozen in liquid nitrogen and stored at −80 °C for future assessment.

Neuronal degeneration described necrotic neurons, morphologically identified with cresyl-violet/acid-fuschin staining as swelling of organelles, loss of cell and nuclear membrane integrity, pyknotic nuclei, bright eosinophilic cytoplasm, or with darkened and condensed cytoplasm (Castillo-Meléndez et al., 2013; Northington et al., 2011). Glial fibrillary acidic protein (GFAP; 1:400, Sigma) immunopositive staining was used to assess the number of astrocytes and identification of activated