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SEIZURES ARE ASSOCIATED WITH BRAIN INJURY SEVERITY IN A NEONATAL MODEL OF HYPOXIA–ISCHEMIA

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Abstract—Hypoxia–ischemia is a significant cause of brain damage in the human newborn and can result in long-term neurodevelopmental disability. The loss of oxygen and glucose supply to the developing brain leads to excitotoxic neuronal cell damage and death; such over-excitation of nerve cells can also manifest as seizures. The newborn brain is highly susceptible to seizures although it is unclear what role they have in hypoxic-ischemic (H/I) injury. The aim of this study was to determine an association between seizures and severity of brain injury in a piglet model of perinatal H/I and, whether injury severity was related to type of seizure, i.e. sub-clinical (electrographic seizures only) or clinical (electrographic seizures+physical signs). Hypoxia (4% O_2) was induced in anaesthetised newborn piglets for 30 min with a final 10 min period of hypotension; animals were recovered and survived to 72 h. Animals were monitored daily for seizures both visually and with electroencephalogram (EEG) recordings. Brain injury was assessed with magnetic resonance imaging (MRI), ^{1}H-MR spectroscopy (^{1}H-MRS), EEG and by histology (haematoxylin and eosin). EEG seizures were observed in 75% of all H/I animals, 46% displayed clinical seizures and 29% sub-clinical seizures. Seizure animals showed significantly lower background amplitude EEG across all post-insult days. Presence of seizures was associated with lower cortical apparent diffusion coefficient (ADC) scores and changes in ^{1}H-MRS metabolite ratios at both 24 and 72 h post-insult. On post-mortem examination animals with seizures showed the greatest degree of neuropathological injury compared to animals without seizures. Furthermore, clinical seizure animals had significantly greater histological injury compared with sub-clinical seizure animals; this difference was not apparent on MRI or ^{1}H-MRS measures. In conclusion we report that both sub-clinical and clinical seizures are associated with increased severity of H/I injury in a term model of neonatal H/I.

Key words: neonatal seizures, hypoxia–ischemia, brain damage.

Moderate to severe hypoxic-ischemic (H/I) encephalopathy has been reported to occur in 3.8/1000 live births and is the most common underlying cause of neonatal seizures (Badawi et al., 1998; Zupanc, 2004). With numerous excitatory circuits and fewer inhibitory circuits, the neonatal brain has a much greater susceptibility to seizures than at any other period in human life (Clancy, 2006). There is significant debate, however, regarding seizures and their involvement in neonatal brain injury (Camfield, 1997; Wasterlain, 1997; Wirrell et al., 2001; Miller et al., 2002). Whilst the immature brain is relatively resistant to seizure-induced damage compared to the adult, there is increasing evidence that seizures are deleterious and can result in long-term neurological deficits (Holmes and Ben-Ari, 1998; Levene, 2002). In animal models, seizures induce perturbations in neurogenesis, neuronal morphology, function and connectivity and neuronal cell death, even in the absence of underlying co-morbidities (Schmid et al., 1999). In human newborns several studies have reported a significant association of seizures with adverse neurodevelopmental outcome (Boylan et al., 1999; Pisani et al., 2008). Miller et al. (Miller et al., 2002) have recently shown a direct association of clinical seizures with severity of brain injury in H/I affected neonates.

Clinical seizures remain the target of clinical management although it is known that more than 60% of neonatal seizures are sub-clinical or "silent", i.e. no physical manifestations (Murray et al., 2007); in 41 neonates using 21 channel electroencephalogram (EEG) 79% of seizures were sub-clinical, only 21% of total EEG seizure time (ictal activity) was associated with clinical seizure activity (Clancy et al., 1988). While misinterpretation of normal movements (mouthing, jitteriness, limb jerks, fisting) may lead to overestimation of clinical seizure activity, studies suggest that limitations in EEG monitoring mean that neonatal seizure frequency especially sub-clinical, is most likely underestimated (Mizrahi and Kellaway, 1987; Malone et al., 2006). However, determining the contribution of neonatal seizures to H/I injury in the developing brain has significant implications for detection and management of seizures in the human newborn, as there is evidence to suggest that currently used anticonvulsant medications may be neurotoxic to the developing brain (Bittigau et al., 2003).

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Abbreviations: ABE, arterial base excess; ADC, apparent diffusion coefficient; ANOVA, analysis of variance; Cho, choline; Cr, creatine; DWI, diffusion weighted image; EEG, electroencephalogram; H/I, hypoxic-ischemic; HCO_3^-, bicarbonate; ^{1}H-MRS, proton magnetic resonance spectroscopy; Lac, lactate; laEEG, low amplitude EEG; MABP, mean arterial blood pressure; MRI, magnetic resonance imaging; NAA, *N*-acetylaspartate; NICU, neonatal intensive care unit; O_2, oxygen; PBS, phosphate-buffered saline; pCO_2, partial pressure carbon dioxide; pO_2, partial pressure oxygen; TE, echo time; TR, repetition time.

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The piglet is a suitable model for the study of H/I injury as its brain growth at birth is comparable to that of the term human newborn (Dobbing and Sands, 1979). Magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (^{1}H-MRS) are used to assess brain structure and function and are the gold standard for evaluating extent of H/I injury in the human newborn. The aim of this study was to determine if there is an association between spontaneous seizures and survivable brain injury in a piglet model of perinatal H/I using two-channel EEG, MRI, ^{1}H-MRS and histology. Furthermore, we determined whether the type of seizure, defined as either sub-clinical (electrographic activity only) or clinical (physical signs+ electrographic activity), was associated with the severity of subsequent H/I brain injury.

EXPERIMENTAL PROCEDURES

Animals

Large White newborn piglets (n=42) were obtained from the University of Queensland Gatton Piggery. Average age and weight was 17.0 h (±1.3) and 1.47 kg (±0.03) respectively. Approval for this study was obtained from the University of Queensland Animal Experimentation Ethics Committee and was carried out in accordance with National Health and Medical Research Council guidelines (Australia). Care was taken to minimize the number of animals used and to ensure no undue pain or distress.

Hypoxia/ischemia

The H/I insult protocol can be found in detail elsewhere (Bjorkman et al., 2006). In brief, hypoxia was induced (n=38) by decreasing inspired oxygen (O_2) to 4% for 30 min and decreased to 2% if low amplitude EEG (laEEG; <5 μV) was not reached within the first 4 min; O_2 was manipulated as necessary to maintain laEEG for the duration of the H/I insult. Hypotension was induced for the final 10 min of the H/I insult by decreasing O_2 until mean arterial blood pressure (MABP) was <70% of baseline. Control animals (n=4) underwent the same protocol excluding the H/I—hypoxic and hypotensive—period. Animals were recovered until euthanasia at 72 h.

Post-insult monitoring—EEG and seizures

Animals were housed in pairs (room temperature 23–25 °C) following recovery from anaesthesia and fed 40–50 ml of artificial pig milk every 3–4 h (Survive Pig Milk Replacer, Think Pig-Country Vet Wholesaling Pty Ltd, VIC, Australia). At each feed, as well as continuously during the 2 h period of EEG monitoring, animals were visually observed for clinical seizure activity and evidence documented for the purposes of seizure classification.

Following the H/I insult on day 0, EEG (BRM2; BrainZ Instruments, Auckland, NZ) was recorded for approximately 2 h each post-insult day on all animals until day 3 using five needle electrodes at C3–P3, C4–P4 as defined by the neonatal modification of the International 10–20 system (Fisch, 1999). The raw EEG traces were analysed off-line using EEG Viewer software (BrainZ Instruments,). Electrographic seizures were defined as repetitive, rhythmic waveforms with a distinct beginning and end with a duration >10 s (Clancy and Legido, 1987; Clancy et al., 1988). Background integrated amplitude was calculated from seizure and artefact free EEG.

Clinical seizures were defined as myoclonic jerks, rhythmic pathologic movements (cycling), or tonic postures. If persistent clinical seizures developed, the animal was treated with anticonvulsant (clonazepam 0.15–0.25 mg/kg). Animals were administered an initial dose and observed for cessation of clinical seizure activity, if after 20 min seizures did not resolve a second dose was given. If clinical seizures did not resolve following a third dose of anticonvulsant, the animal was euthanased as required by the University of Queensland ethics committee. Animals with subclinical seizures (electrographic only) were not treated.

Neurobehavioural scoring

Animals were assessed for neurobehaviour at 8, 12, 16, 24, 48 and 72 h as previously described (Thoresen et al., 1996; Foster et al., 2001). Animals were assessed on nine neurologic measures such as level of consciousness, respiration, ability to stand and walk, the righting reflex and presence of clinical seizures as described above (see post-insult monitoring). Each neurologic measure was assigned a score of 2 (normal), 1 (moderately abnormal) or 0 (pathologic). The nine neurologic measures were totalled to achieve a maximal score of 18=normal.

Magnetic resonance imaging

MRI head scans were performed on post-insult days 1 and 3. MRI data were acquired using a 4 T Bruker/siemens whole body scanner with a custom-built knee extremity coil. Sagittal and coronal high-resolution T_2-weighted anatomical images were acquired using a multi-echo, fast spin echo sequence with the following parameters: six contiguous 4 mm slices, repetition time/(echo time) (TR/(TE)) 1500/(74/147/221) ms, FOV 120 mm, acquisition matrix 256×205, image matrix 256×256 and turbo factor of five. A series of multi-slice coronal diffusion weighted images (DWI) were acquired at the same slice position as the high-resolution anatomical T_2-weighted coronal images. The DWI were acquired with the following parameters: TR/TE 2500/80 ms, FOV 120 mm, six slices, 4 mm slice thickness, acquisition matrix 128×64, image matrix 128×128, with 4 b values (0, 350, 750 and 1000 s/mm^2). The apparent diffusion coefficient (ADC) and T_2-weighted maps were generated using the Siemens image analysis software (Erlangen, Germany). Average ADC values for cortical and subcortical regions were used to allow comparison across animals as previously reported (Moxon-Lester et al., 2007). Animals that required sedation were administered Zolteil (Tiletamine/Zolazepam 10 mg/kg, Virbac, NSW, Australia).

^{1}H-magnetic resonance spectroscopy

^{1}H-MR spectra was obtained on a 4 T Bruker/Siemens as described above. A single spectrum was acquired on post-insult days 1 and 3 in the fronto-parietal region of the brain from a 15 mm^3 voxel using point-resolved spectroscopy with the following parameters: TR=1500 ms, TE=136 ms, and 192 averages. Metabolite spectra were exported, processed and analysed using the AMARES tool within jMURI and manually phased (Vanhamme et al., 1997). Prior to quantification, apodisation (10 Hz) was applied to the *N*-acetylaspartate (NAA), creatine (Cr) and choline (Cho) peaks with Lorentzian peaks. Lactate (Lac) was represented as an inverted broad peak (no doublet character) and thus was quantified 180° out of phase with the other metabolite peaks using a Gaussian peak. Peak area ratios were calculated for NAA/Lac, NAA/Cho, NAA/Cr, Lac/Cr, Lac/Cho, Lac/NAA and Cho/Cr. Animals requiring sedation were administered Zoletil i.m. (10 mg/kg, Virbac).

Tissue collection

Animals were euthanased via an intracardiac injection of Lethabarb (650 mg/kg) at 72 h post-insult. The brain was removed, coronally sectioned (3–4 mm) and fixed in 4% paraformaldehyde/0.1 M phosphate buffered saline pH 7.2 (PBS) for 24 h with agitation followed by transfer into 10% Picric Acid/PBS for a further 8–12 h. The brain sections were stored in PBS/sodium azide (0.05%).