Research using Animal Models to Improve Care of Neonatal Encephalopathy

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Research using Animal Models to Improve Care of Neonatal Encephalopathy

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Abstract
Animal models have become indispensable in the search for novel strategies and therapeutics for effective therapies for neonatal encephalopathy (NE). Over the last 3 decades, the translation of ideas and questions from bedside to bench moved therapeutic hypothermia into the clinic as the first safe and effective therapy for NE. We are now at the stage where animal models are needed to move therapies further forward. We review some of the main animal models in use and discuss advantages and disadvantages of each model, giving a detailed example of a protocol used in a piglet model of NE.

Introduction
NE is responsible for a significant burden of disability and death worldwide (1). The use of animal models in the study of perinatal hypoxia-ischaemia has a history of over 200 years; studies initially showed that the premature animal is more tolerant of asphyxia than a term animal which in turn more resistant to asphyxia than an adult (2, 3). In the 1950s-1970s, studies in the primate model showed that the pattern of brain injury was clearly influenced by the severity and type of hypoxia-ischaemia; these studies led to a description of two patterns of injury, namely: acute total asphyxia (4) and chronic partial asphyxia (5). In the last 30 years progress was made by using animal models of NE to understand the timing of the evolution brain injury after hypoxia-ischaemia. The translation of ideas and questions from bedside to bench moved therapeutic hypothermia into the clinic as the first safe and effective therapy for NE (6) (Fig 1). Triggered by the observation in human babies that brain energy metabolism on phosphorus-31 ($^{31}$P) magnetic resonance spectroscopy (MRS) transiently recovered after birth and declined in the hours and days after birth despite intensive care support (7), studies in the newborn piglet (8) and rat (9) allowed pathophysiology and timing of events after hypoxia-ischaemia to be studied more precisely than in the human fetus and neonate. The concept that a therapy started after the injury changed the trajectory of brain damage was established.

Factors to consider for translation of animal studies to humans
There has been recent criticism of animal research (10) because of overoptimistic conclusions and inadequate control of bias especially in relation to compounds which, in reality, have little or no therapeutic potential. There are calls for more systematic reviews of animal studies and higher standards of research conduct and reporting (10). Dobbing and Sands used rates of brain growth to make cross species comparisons and provided the foundation for the use of animal models to study
neonatal brain injury (11). Such models need to take into account the timing of the brain growth spurt in relation to the injury and whether the animal is *altricial*, born at a relatively underdeveloped stage with many neurogenic events occurring postnatally (rodents, cats, dogs, rabbits, humans) or *precocial*, where young are relatively mature and mobile from the moment of birth (guinea pig, rhesus monkey, piglet, sheep). This is important because cellular and regional vulnerability of brain varies by developmental stage. Differences in brain complexity and white to gray matter ratios are also important factors (12). The rodent brain is lissencephalic with a much smaller proportion of white matter than in humans, a different focus of neurogenesis and timing of myelination. The rate of rodent maturation is accelerated compared to humans with each day of development in a rat corresponding to more than a week in humans (13). However in studies of acute hypoxia-ischaemia the timing of the cellular response to injury appears to be similar in rodents and humans. Examples of studies relevant to NE are summarized in the table.

**Rodents**

The Rice-Vannucci rodent model was established in 1981 (14) and remains one of the most commonly used models of neonatal hypoxic ischaemia (HI). Rat pup brains on postnatal day (P) 7 and 10 are developmentally similar to a 32 week gestation and term human infant respectively. The classic Rice-Vannucci model involves unilateral carotid artery ligation and subsequent inhalation of 8% oxygen for 90 minutes. Since its initial description this model has been adapted to younger and older rats and mice, showing the different vulnerabilities of different ages and species (15). This model has considerable injury variability and produces damage to both white and gray matter.

**Sheep**

Several models of *in utero* hypoxia-ischemia in fetal lambs have been developed, including exposure to maternal hypoxemia umbilical cord occlusion at different gestations (16, 17), or bilateral carotid artery occlusion (18). In landmark studies in the 1990s Gunn and colleagues utilised near term fetal sheep to evaluate the pathophysiology of NE. Romney/Suffolk sheep between 117 to 124 days (77-82%) gestation were instrumented with umbilical catheters, carotid artery occluders and intra-cerebral EEG probes before being returned to the intact uterus. Cerebral ischemia was induced by carotid occlusion and confirmed by EEG. Cytotoxic cell swelling and accumulation of excitotoxins were observed. Gunn and colleagues later evaluated the efficacy of selective head cooling by inserting a scalp cooling coil at
the time of surgery. They demonstrated that 72 hours of hypothermia reduced
cortical cytotoxic oedema and reduced cortical infarction and neuronal loss (18).

Non-Human Primate
Ranck and Windle (4) and Myers (5) developed the monkey models of NE in the
1950s to 1970s. Acute total asphyxia was induced at term by detaching the placenta
at hysterotomy. Eleven to 16 minutes later the fetuses were delivered from their
membranes and resuscitated (4). The pattern of injury was very similar to that seen
in infants with a basal ganglia and thalamus injury following a sentinel event (19). In
the partial asphyxia model, intrauterine asphyxia was produced in the pregnant term
monkey by breathing halothane to cause maternal hypotension. Each fetus was
exposed to asphyxia for 1-5 hours and then delivered and resuscitated and ventilated
for 2 days. The pattern of injury was parasagittal, affecting the watershed areas (5).
A primate model is currently used in neonatal neuroprotection research where cord
occlusion is for 15-18 minutes with extended MRI, MRS and neurodevelopmental
follow-up to 9 months (20).

Piglet
Pigs are a suitable non-primate models of NE due to their relatively large size and
anatomical similarities to man. Unlike rodents, their brain size more closely reflects
the white/gray matter ratio seen in humans and is at comparatively similar
developmental age at term (21). Pigs express similar metabolic changes as seen
with MRS in asphyxiated infants. At UCL we use bilateral carotid artery occlusion in
combination with hypoxia over 25 minutes to assess efficacy and safety of
neuroprotective interventions (22-24). We quantify the hypoxic-ischaemic insult using
$^{31}$P MRS, enabling standardisation across groups and smaller group sizes. A typical
experimental protocol is shown in Fig 2. Outcome biomarkers to assess safety and
efficacy of interventions are similar to human neonates, for example EEG, $^{31}$P and $^1$H
MRS as well as immunohistochemistry and gene expression studies.

Conclusion
Animal models have been central to the development of neuroprotective
interventions for the human neonate in the last 25 years. The routine use of
therapeutic hypothermia for moderate to severe NE was a significant milestone,
however further work is needed as, despite treatment, 25% of untreated infants die
and 20% of survivors have sensorimotor and cognitive deficits. Further work is
needed to evaluate the efficacy of neuroprotective drugs both as single agents as
well as in combination. Studies in small animal models will be required to define optimal combinations and timing of therapies before moving down the pathway of more complex animal models prior to human studies. The sensitising role of infection and inflammation is recognised as an important factor and specific neuroprotective agents may be needed to maximise protection. The “neuroprotection Holy Grail” may lie in uncovering these different disease phenotypes and employing a combination of therapeutic strategies to maximise clinical benefit.
References


<table>
<thead>
<tr>
<th>Animal</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Examples of research findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venetucci</td>
<td>• Inexpensive and experiments can involve large numbers</td>
<td>• Small body size makes it difficult to accurately monitor multiple organ function</td>
<td>Assessing neuroprotective impact of manipulating physiological parameters</td>
</tr>
<tr>
<td>Rice</td>
<td>• At 7 days, CNS maturation considered to be comparable to neonatal infant</td>
<td>• Postnatal CNS maturation is rapid</td>
<td>- Hypoglycaemia (deleterious)</td>
</tr>
<tr>
<td>Rodent</td>
<td>• Extensive literature on baseline neurochemistry and behavioural assessment</td>
<td>• Rat brain may be more resistant to hypoxic ischaemic insults than human newborn brain</td>
<td>- Hypothermia (very protective)</td>
</tr>
<tr>
<td></td>
<td>• Suitable for short and long term experimental outcomes</td>
<td></td>
<td>- Hypoxic preconditioning (protective)</td>
</tr>
<tr>
<td>Non-human</td>
<td>• Closest phylogenetic origin to human</td>
<td>• Very expensive model</td>
<td>Evaluation of pathophysiological processes during HI, e.g. impact on the blood brain barrier,</td>
</tr>
<tr>
<td>primate</td>
<td>• Body size sufficient to perform accurate physiological monitoring</td>
<td>• Unresolved ethical concerns in the use of higher order animal species</td>
<td>cerebral blood flow and regional cerebral glucose utilization</td>
</tr>
<tr>
<td></td>
<td>• Model of HI can incorporate short term physiological and biochemical</td>
<td>• CNS of baboons and rhesus monkeys are more mature than a human newborn</td>
<td></td>
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<tr>
<td></td>
<td>outcomes as well as long-term behavioural outcomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piglet</td>
<td>• Well established model with data on cerebral metabolic processes and</td>
<td>• Animals mature rapidly, thus accuracy of postnatal age is important</td>
<td>Different patterns of injury are associated with the degree and duration of hypoxia/anoxia</td>
</tr>
<tr>
<td></td>
<td>histological analysis</td>
<td>• Typical HIE syndrome may be difficult to reproduce consistently</td>
<td>as well as the presence of acidosis</td>
</tr>
<tr>
<td></td>
<td>• CNS maturation of piglet &lt; 48 hours age is comparable to human neonate</td>
<td>• Mostly suited to short term analysis as long term neurological outcome data is not well</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Body size sufficient to perform accurate physiological monitoring</td>
<td>established</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Relatively inexpensive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep /</td>
<td>• Hypoxic ischaemia can be administered on catheterized lamb fetus in utero</td>
<td>• Availability is restricted in some laboratories due to concerns regarding Q fever</td>
<td>Cooling ameliorates secondary energy failure with a reduction in excitatory amino acid and</td>
</tr>
<tr>
<td>Lamb</td>
<td>without prior anaesthesia</td>
<td></td>
<td>nitric oxide release</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cooling reduces degree of apoptosis rather than necrotic cell death</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary energy failure is accompanied by cytotoxic cellular oedema and excitotoxin release</td>
</tr>
<tr>
<td>Advantages</td>
<td>Limitations</td>
<td>Selective head cooling for 72 hours is safe and dramatically reduced cortical infarction and neuronal loss(^{19})</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
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</tbody>
</table>
| • Body size sufficient to perform accurate physiological monitoring  
• Relatively inexpensive                              | • CNS of lambs are more mature than human newborns\(^{26}\)  
• Mostly suited to short term analysis as long term neurological outcome data is not well established |                                                                                  |

Table 1. The advantages and limitations of commonly used animal models
Bench

1950-1970

Different brain injury patterns were defined for acute total and partial asphyxia in the monkey (Ravick and Windle 1959; Myers 1972)

1980

The Rice-Vannucci model is established

1995

Piglet model demonstrates that hypothermia ameliorates brain energy decline on MRS (Thoresen 1995)

1997

Gunn and colleagues show that 72°C hypothermia is safe and effective in reducing cytotoxic oedema (Gunn, 1997)

2005

Randomised control trials demonstrate that cooling reduces mortality without increasing disability in survivors of perinatal hypoxia ischaemia

2010

NICE National Institute for Health and Care Excellence
National guidance introduced in May 2010 and therapeutic hypothermia becomes the standard treatment of moderate to severe neonatal encephalopathy

Bedside

2020

The Future?
Tailoring therapies to individual patients based on injury severity, gender, inflammatory status and genetics.

2020

Cooling adjuncts...
Combination of agents can be evaluated in more complex animal models prior to human trials

2016

Further work
More data is needed to define the optimum combination and timing of new neuroprotective strategies
The piglet undergoes a thorough clinical examination, ensuring it is active, healthy and an appropriate weight for a newborn (1.8-2.2 kg).

**1. Health Check**

Baseline recordings are taken on the piglet’s physiological parameters (HR, RR, Temp, BP) and amplitude integrated EEG. The NIRS probes are sited and calibrated. Prior to the hypoxia-ischaemia event, baseline lactate/NAA is measured using 1H MRS.

The piglet is anaesthetised with isofluorane via a facemask with monitoring of oxygen saturations. Isoflurane inhalation and fentanyl infusion are continued throughout the study and the piglet is insentient throughout. A tracheostomy is sited and the piglet is connected to a ventilator. An umbilical arterial catheter is inserted for blood sampling and central arterial BP monitoring and an umbilical venous catheter for fluid and drug administration.

**2. Surgery**

The piglet is ventilated with a nitrogen / air mix (4-6% oxygen) for 45 minutes. The degree of hypoxia is titrated to physiological parameters and fall in cytochrome activity on NIRS. EEG recordings are taken continuously and the HI insult is quantified by the duration of time the EEG flattens below 7 microvolts. 31P MRS during this period of time demonstrates the reduction in high-energy phosphate activity and increase in inorganic phosphate.

**3. Baseline Measurements and Neuroimaging**

Baseline recordings are taken on the piglet’s physiological parameters (HR, RR, Temp, BP) and amplitude integrated EEG. The NIRS probes are sited and calibrated. Prior to the hypoxia-ischaemia event, baseline lactate/NAA is measured using 1H MRS.

**4. Hypoxia-ischaemia**

The piglet is ventilated with a nitrogen / air mix (4-6% oxygen) for 45 minutes. The degree of hypoxia is titrated to physiological parameters and fall in cytochrome activity on NIRS. EEG recordings are taken continuously and the HI insult is quantified by the duration of time the EEG flattens below 7 microvolts. 31P MRS during this period of time demonstrates the reduction in high-energy phosphate activity and increase in inorganic phosphate.

**5. Evaluation over 48 hours**

<table>
<thead>
<tr>
<th>Time</th>
<th>HI</th>
<th>MRI</th>
<th>MRI</th>
<th>Continues EEG monitoring</th>
<th>Treatment of hypotension/seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>24</td>
<td>48</td>
<td></td>
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</tbody>
</table>

The piglet is managed in an intensive care environment for a further 48 hours and treated for complications of HIE (eg. hypotension, seizures, hyperkalemia). The progress of the piglet is monitored by continuous EEG and serial MR scans at 24 and 48 hours following the sentinel event.

**6. Post-Mortem**

After 48 hours, the piglet is euthanised and undergoes a post-mortem. The internal organs are visually inspected for signs of hypoxia-ischaemia. The brain is fixed and dissected for histological examination to determine the extent of neuronal injury at specific regions of interest.

Key

1H MRS: Proton Magnetic Resonance Spectroscopy
31P MRS: Phosphorus Magnetic Resonance Spectroscopy
HI – Hypoxic Ischaemia
HR = Heart rate, RR = Respiratory Rate, Temp = Temperature,
BP – Blood pressure
EEG – Electroencephalogram
NAA – N acetyl aspartate
NIRS – Near Infrared Spectroscopy