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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 is 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 ischaemia 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.

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Animal	Advantages	Limitations	Examples of research findings
Vennucci Rice Rodent	• Inexpensive and experiments can involve large numbers • At 7 days, CNS maturation considered to be comparable to neonatal infant[15] • Extensive literature on baseline neurochemistry and behavioural assessment[16,26] • Suitable for short and long term experimental outcomes	• Small body size makes it difficult to accurately monitor multiple organ function • Postnatal CNS maturation is rapid • Rat brain may be more resistant to hypoxic ischaemic insults than human newborn brain[26]	Assessing neuroprotective impact of manipulating physiological parameters[27] -Hypoglycaemia (deleterious) -Hypothermia (very protective) -Hypoxic preconditioning (protective) Evaluation of pathophysiological processes during HI, e.g. impact on the blood brain barrier, cerebral blood flow and regional cerebral glucose utilization[27]
Non-human primate	• Closest phylogenic origin to human[28] • Body size sufficient to perform accurate physiological monitoring • Model of HI can incorporate short term physiological and biochemical outcomes as well as long-term behavioural outcomes	• Very expensive model • Unresolved ethical concerns in the use of higher order animal species • CNS of baboons and rhesus monkeys are more mature than a human newborn[28]	Different patterns of injury are associated with the degree and duration of hypoxia/anoxia as well as the presence of acidosis[5]
Piglet	• Well established model with data on cerebral metabolic processes and histological analysis • CNS maturation of piglet < 48 hours age is comparable to human neonate[29] • Body size sufficient to perform accurate physiological monitoring • Relatively inexpensive	• Animals mature rapidly, thus accuracy of postnatal age is important • Typical HIE syndrome may be difficult to reproduce consistently[26] • Mostly suited to short term analysis as long term neurological outcome data is not well established	Cooling ameliorates secondary energy failure[30] with a reduction in excitatory amino acid and nitric oxide release[31] Cooling reduces degree of apoptosis rather than necrotic cell death[32]
Sheep / Lamb	• Hypoxic ischaemia can be administered on catheterized lamb fetus in utero without prior anaethesia[19,26]	• Availability is restricted in some laboratories due to concerns regarding Q fever	Secondary energy failure is accompanied by cytotoxic cellular oedema and excitotoxin release[33]

	• 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	Selective head cooling for 72 hours is safe and dramatically reduced cortical infarction and neuronal loss[19]

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

Key Milestones

Bench → Bedside

1950-1970

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

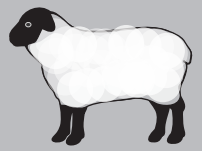
1980

The Rice-Vannucci model is established

1995

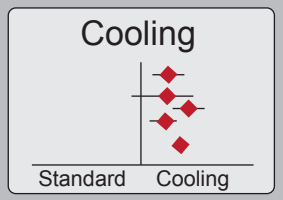
Piglet model demonstrates that hypothermia ameliorates brain energy decline on MRS (Thoresen 1995)

1997



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

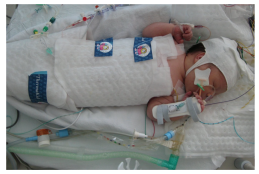
2005



Randomised control trialsdemonstrate that cooling reduces mortality without increasing disability in survivors of perinatal hypoxia ishaemia

Gluckman Lancet 2005
Shankaran NEJM 2005
Azzopardi NEJM 2010

2010



NICE National Institute for Health and Care Excellence

National guidance introduced in May 2010 and therapeutic hypothermia becomes the standardtreatment of moderate to severe neonatal encephalopathy

Bench ← Bedside

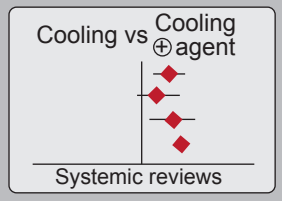
2016

Further work

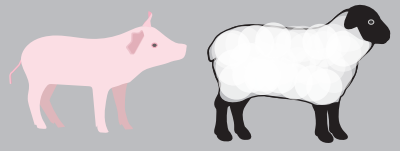
More data is needed to define the optimum combination and timing of new neuroprotective strategies

2020

Cooling adjuncts…



The future…



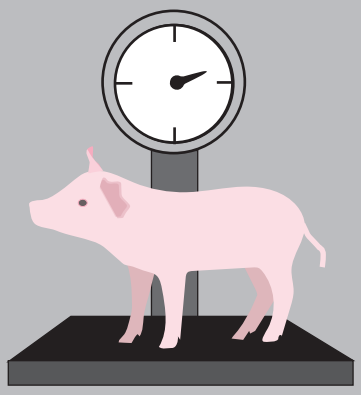
Combination of agents can be evaluated in more complex animal models prior to human trials

2020

The Future ?

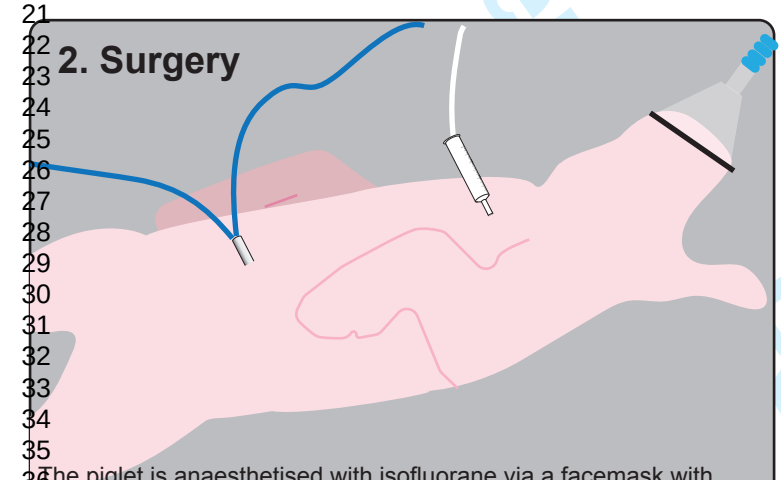
Tailoring therapies to individual patients based on injury severity, gender, inflammatory status and genetics.

1. Health Check



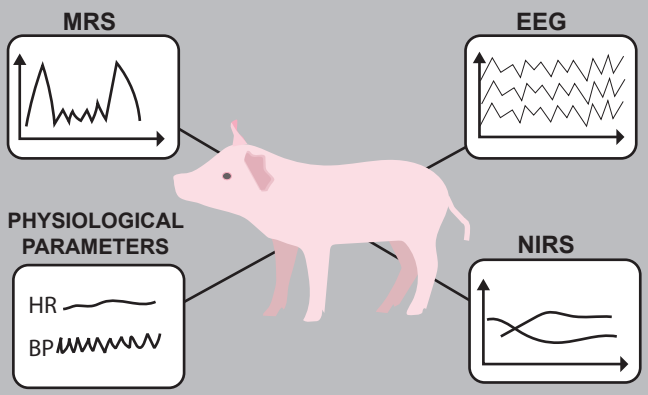
The piglet undergoes a thorough clinical examination, ensuring it is active, healthy and an appropriate weight for a newborn (1.8-2.2 kg)

2. Surgery



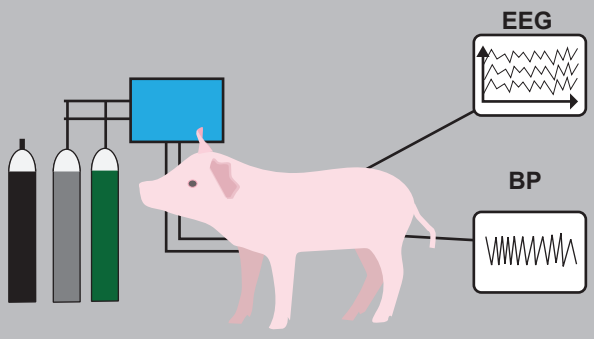
The piglet is anaesthetised with isofluorane via a facemask with monitoring of oxygen saturations. Isoflurance inhlataion and fentanyl infusion are continued throughout the study and the piglet is insentient throughout. A tracheostomy is sited and the piglet is connected to a ventilator. An umbilical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

3. Baseline Measurements and Neuroimaging



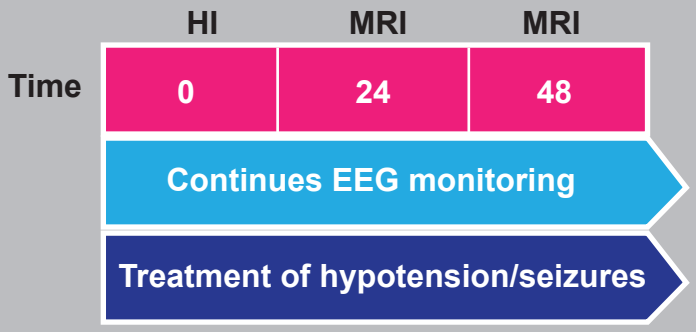
Baseline recordings are taken on the piglet's physiological parameters (HR, RR, Temp, BP) and amplitude integrated EEG. The NIRS probes are sited and calibrated. Prior to the hypoxia-ischaemia event, baseline lactate/NAA is measured using 1H MRS.

4. Hypoxia-ischaemia



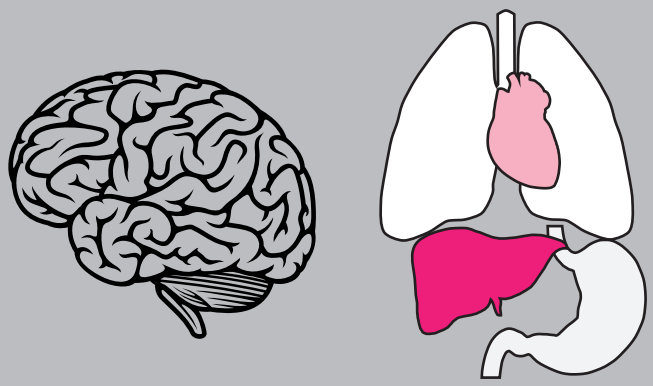
The piglet is ventilated with a nitrogen / air mix (4-6% oxygen) for 45 minutes. The degree of hypoxia is titrated to physiological parameters and fall in cytochrome activity on NIRS. EEG recordings are taken continuously and the HI insult is quantified by the duration of time the EEG flattens below 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



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