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Inhaled 45–50% argon augments hypothermic brain protection in a piglet model of perinatal asphyxia

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ABSTRACT

Cooling to 33.5 °C in babies with neonatal encephalopathy significantly reduces death and disability, however additional therapies are needed to maximize brain protection. Following hypoxia–ischemia we assessed whether inhaled 45–50% Argon from 2–26 h augmented hypothermia neuroprotection in a neonatal piglet model, using MRS and aEEG, which predict outcome in babies with neonatal encephalopathy, and immunohistochemistry. Following cerebral hypoxia–ischemia, 20 Newborn male Large White piglets < 40 h were randomized to: (i) Cooling (33 °C) from 2–26 h (n = 10); or (ii) Cooling and inhaled 45–50% Argon (Cooling + Argon) from 2–26 h (n = 8). Whole-brain phosphorus-31 and regional proton MRS were acquired at baseline, 24 and 48 h after hypoxia–ischemia. EEG was monitored. At 48 h after hypoxia–ischemia, cell death (TUNEL) was evaluated over 7 brain regions. There were no differences in body weight, duration of hypoxia–ischemia or insult severity; throughout the study there were no differences in heart rate, arterial blood pressure, blood biochemistry and inotrope support. Two piglets in the Cooling + Argon group were excluded. Comparing Cooling + Argon with Cooling there was preservation of whole-brain MRS ATP and PCr/Pi at 48 h after hypoxia–ischemia ($p < 0.001$ for both) and lower ^{1}H MRS lactate/N acetyl aspartate in white ($p = 0.03$ and 0.04) but not gray matter at 24 and 48 h. EEG background recovery was faster ($p < 0.01$) with Cooling + Argon. An overall difference between average cell-death of Cooling versus Cooling + Argon was observed ($p < 0.01$); estimated cells per mm^2 were 23.9 points lower (95% C.I. 7.3–40.5) for the Cooling + Argon versus Cooling. Inhaled 45–50% Argon from 2–26 h augmented hypothermic protection at 48 h after hypoxia–ischemia shown by improved brain energy metabolism on MRS, faster EEG recovery and reduced cell death on TUNEL. Argon may provide a cheap and practical therapy to augment cooling for neonatal encephalopathy.

1. Introduction

Neonatal Encephalopathy (NE) consequent on perinatal hypoxia–ischemia is the third leading cause of child death and one of the main causes of preventable child neurodisability worldwide (Lawn et al., 2014). In the developed world, cooling to 33–34 °C for 72 h in moderate to severe NE increases the rate of survival without impairments in childhood to 15%, but despite cooling, around 25% infants die and 20% survivors have sensorimotor or cognitive impairments (Azzopardi et al., 2014). Attempts to increase brain protection with deeper and longer cooling (Alonso-Alconada et al., 2015; Shankaran et al., 2014) suggest that current clinical cooling protocols are optimal and that other therapies that can augment hypothermic neuroprotection in NE are needed (Robertson et al., 2012).

In a comparative review of potential neuroprotective agents, the noble gas Xenon was rated in the top six, however there was concern over its cost and the requirement for specialized equipment for delivery

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and scavenging (Robertson et al., 2012). Xenon has shown neuroprotective properties in adult and neonatal (Chakkarapani et al., 2010; Faulkner et al., 2011; Ma et al., 2005) models of hypoxia–ischemia; this neuroprotection is stronger in neonatal models when Xenon is combined with cooling. In neonatal rat (Ma et al., 2005) and piglet (Chakkarapani et al., 2010; Faulkner et al., 2011) studies, the combination of Xenon with cooling provided neuroprotection while neither intervention alone was as effective. Current interest is turning towards Argon, which is the most abundant inert gas already widely used in industries and available at a cost 200 times lower than Xenon. Argon does not produce demonstrable anesthetic effects at atmospheric pressure and provides potent neuroprotection, at least equivalent to Xenon, in animal models of hypoxic–ischemic brain injury and *in vitro* using murine organotypic hippocampal slice cultures (Loetscher et al., 2009) and neuronal cultures (Jawad et al., 2009). In some studies Argon is superior to Xenon for organ protection from ischemia-reperfusion injury (Irani et al., 2011). *In vitro* models of cerebral ischemia and traumatic brain injury suggest that the optimum concentration of Argon for protection is 50% and the therapeutic window lasts up to 3 h (Loetscher et al., 2009). Argon 50% administered one hour after transient middle cerebral artery occlusion (MCAO) in adult rats provided significantly reduced infarct volumes and composite adverse outcomes (Ryang et al., 2011). Protection has also been observed in neonatal rodent models where 70% argon at 2 h after hypoxia–ischemia improved cell survival to naive levels and reduced infarct volume (Zhuang et al., 2012).

We hypothesized that Argon-augmented cooling would lead to better brain protection than cooling alone after a hypoxic–ischemic insult. Our aim was to assess whether 24 h of 45–50% Argon started 2 h after hypoxia–ischemia augments hypothermic neuroprotection in a piglet perinatal asphyxia model. This model replicates neonatal intensive care with meticulous monitoring and control of physiological and metabolic parameters. This model also has strong similarities to newborn infants with NE in terms of the timing of the evolution of injury after hypoxia–ischemia (Azzopardi et al., 1989; Lorek et al., 1994), pattern of injury, neuropathology and cerebral magnetic resonance spectroscopy (MRS) (Thayyil et al., 2010). The efficacy of Argon protection was assessed using: (i) Cerebral MRS biomarkers, proton (^{1}H) MRS lactate/N acetyl aspartate (NAA) (Thayyil et al., 2010) and phosphorus-31 (^{31}P) MRS for phosphocreatine/inorganic phosphate (PCr/Pi) and NTP/exchangeable phosphate pool (epp) (Azzopardi et al., 1989); (ii) aEEG background activity recovery over 48 h, a strong predictor of outcome in babies with NE (van Rooij et al., 2005); and (iii) Histological assessment of cell death using TUNEL at 48 h after hypoxia–ischemia.

2. Materials and methods

2.1. Sample size calculation

Our primary outcomes were cerebral lactate/NAA and NTP/epp. Previous work with our model suggested that the change in lactate/NAA during 48 h varied between normo- and hypothermic groups by 1.0 U, with a standard deviation of 0.65 U (log scale). Assuming similar magnitude of additional effect for Argon-augmented cooling following HI versus cooling alone and similar variability at 48 h and with 5% significance and 80% power, at least eight subjects were required in each group based on a two-sample t-test sample size calculation.

2.2. Animal experiments and surgical preparation

All animal experiments were approved by the Ethics Committee of UCL and performed according to the UK Home Office Guidelines [Animals (Scientific procedures) Act, 1986]. The study complies with the ARRIVE guidelines. Twenty male piglets, aged less than 40 h, with weights 1.8–2.1 kg were anesthetized and surgically prepared as described previously (Lorek et al., 1994). The study time-line is shown in Fig. 1. Anesthesia was induced by 4% *v/v* isoflurane through a facemask for around 5 min to facilitate tracheostomy and intubation. Throughout the surgery, isoflurane was maintained at 2.8–3% guided by peripheral oxygen saturation monitoring (Nonin Medical, Plymouth, MN, USA) and the animal's response to stimulation. Following tracheostomy, a suitable size of endotracheal tube (Smiths Medical, Ashford, Kent, UK) was fixed and the piglet was mechanically ventilated (SLE 2000 infant ventilator, Surrey, UK). Ventilator settings were adjusted to maintain partial pressure of oxygen (PaO_2) at 8–13 kPa and carbon dioxide ($PaCO_2$) at 4.5–6.5 kPa, allowing for temperature and fraction of inspired oxygen (FiO_2) correction of the arterial blood sample.

After the airway was secured, both common carotid arteries were surgically isolated at the level of the fourth cervical vertebra and a vascular occluder (OC2A, In Vivo Metric, Healdsburg, CA, USA) was placed on each side. After completion of surgery, inspired isoflurane concentration was maintained at 2% v/v.

An umbilical venous catheter was inserted for infusion of maintenance fluids (10% dextrose, 60 ml/kg/day before the insult and 40 ml/kg/day after resuscitation), fentanyl (5 μg/kg/h), and antibiotics (benzyl penicillin 50 mg/kg, every 12 h and gentamicin 4 mg/kg, once a day). An umbilical arterial catheter was inserted for invasive physiologic monitoring (SA instruments) for heart rate and arterial blood pressure, and blood sampling for arterial gases and electrolytes (Abbot Laboratories, UK). Hepsal (0.5 IU/ml of heparin in 0.9% saline solution) was infused at rate of 0.3 ml/h to prevent umbilical arterial catheter blockage.

Piglets were cared for under intensive care conditions throughout the experiment. To maintain the MABP above 40 mm Hg, bolus infusions of 0.9% saline (Baxter; 10 ml/kg), dopamine (5–20 μg/kg/min), dobutamine (5–20 μg/kg/min) and adrenaline (0.1–1.5 μg/kg/min) were used as required by a NICU trained clinician. High serum lactate was treated by optimizing oxygenation and 0.45% saline bolus infusions. Hyperkalemia ($K > 7.0$ mmol/l) was treated with 4 μg/kg salbutamol (10 μg/ml) over 10 min.

2.3. MR methods

The head was immobilized in a stereotactic frame for MRS acquisition. Piglets were positioned within the bore of 9.4 Tesla Agilent MR scanner. ^{1}H and ^{31}P MRS data were acquired at baseline and at 24 and 48 h after cerebral hypoxic-ischemia.

2.3.1. ^{31}P MRS

A 7 cm × 5 cm elliptical transmit-receive MRS surface coil tuned to the ^{31}P resonant frequency was positioned on top of the head. ^{31}P MRS was acquired with 1-minute resolution using a non-localized single-pulse acquisition. MRS data were analyzed using the Advanced Method for Accurate, Robust and Efficient Spectral fitting of MRS data with use of prior knowledge (AMARES) (Vanhamme et al., 1997) as implemented in the jMRUI software. Prior knowledge of NTP multiplet structure was used. NTP is predominately ATP and the latter contributes approximately 70% of the NTP signal (Mandel and Edel-Harth, 1966). Thus NTP changes during this experiment predominately reflected ATP changes. Pi was fitted using 4 separate components and PCr with a single component. The following peak-area ratios were calculated: Pi/epp, PCr/epp, and NTP/epp where epp = exchangeable phosphate pool = Pi + PCr + 2γ-NTP + β-NTP.

2.3.2. ^{1}H MRS

^{1}H MRS data were collected from voxels located in the dorsal right subcortical white matter at the centrum semiovale level (white matter voxel, 8 × 8 × 15 mm) and in the deep gray matter centered on both lateral thalami (deep gray matter voxel, 15 × 15 × 10 mm) using a combination of a 65 × 55 mm elliptical receive surface coil, a 150 mm diameter transmit volume coil and a LASER acquisition (TR = 5000 ms, TE = 288 ms, 128 averages). Spectra were analyzed using AMARES as implemented in the jMRUI software and the lactate/NAA peak area ratio was calculated.