Short-term effects of Early Initiation of Magnesium Infusion combined with Cooling after Hypoxia-Ischemia in Term Piglets

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The manuscript was authored by IL and edited by NJR. Experiments performed by IL, CM, AAB and KM. Immunohistochemistry staining undertaken by MH; counting performed by IL, CM and KM. NIRS data collected with support from PK, CB and IT. MRS data acquisition led by XG. All co-authors have reviewed this manuscript.

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Article Impact Questions:

- $MgSO_4$ bolus and infusion is a safe regimen to raise and maintain supraphysiological serum magnesium levels during hypothermic neuroprotection.

- Doubling serum magnesium resulted in a modest 16% rise in CSF magnesium after a 48h infusion.

- Mg+HT therapy significantly reduced overall neuronal cell death and increased numbers of myelin-producing oligodendrocytes.

- The lack of efficacy seen on aEEG or MRS biomarkers suggest benefit is incremental and therefore unlikely to translate into substantive improvement in clinical trials.

- Further pre-clinical study of magnesium is necessary to evaluate its effectiveness as part of a cocktail of complementary neuroprotective therapies.

Abstract

Introduction: Neuroprotection from therapeutic hypothermia (HT) is incomplete, therefore additional strategies are necessary to improve long-term outcomes. We assessed the neuroprotective efficacy of magnesium sulphate ($MgSO_4$) bolus and infusion over 48h plus HT in a piglet model of term neonatal encephalopathy (NE).

Methods: 15 newborn piglets were randomized following hypoxia ischemia (HI) to: (i) $MgSO_4$ 180mg/kg bolus and 8mg/kg/h infusion with HT (Mg+HT); or (ii) HT and saline 0.5ml/h (HT). Treatments were initiated 1h post-HI; HT administered for 12h (33.5^oC). HI was performed by transient carotid occlusion and inhalation of 6%O_2 for 20-25min. Primary outcomes included aEEG, magnetic resonance spectroscopy (MRS) at 24h, 48h and immunohistochemistry.

Results: $MgSO_4$ bolus and infusion was well tolerated (no hypotension) and doubled serum magnesium (0.72 vs 1.52mmol/L) with modest (16%) rise in CSF. In Mg+HT compared to HT, there was overall reduced cell death (p=0.01), increased oligodendrocytes (p=0.002). No improvement was seen on aEEG recovery (p=0.084) or MRS (Lac/NAA; PCr/Pi; NTP/epp) (p>0.05) at 48h.

Conclusion: Doubling serum magnesium with HT was safe, however the small incremental benefit of Mg+HT compared to HT is unlikely to translate into substantive long-term improvement. Such an incremental effect might justify further study of Mg in combination with multiple therapies.

Neonatal encephalopathy (NE) represents a significant global health burden and is the second leading cause of mortality in infants under 28 days (1). Therapeutic hypothermia (HT) is currently the only routine neuroprotective strategy shown to be effective in intensive care settings, however mortality and morbidity remain high at almost 50% despite treatment (2). Optimizing HT by cooling to lower temperatures (32^{o}C) and for longer duration (120h) failed to improve neurological outcomes (3) and attention is now directed towards adjunct pharmacological agents.

Magnesium sulphate ($MgSO_4$) is a cheap and widely available drug with a well-documented side effect profile. It has recently been shown to reduce the incidence of cerebral palsy in preterm infants when administered antenatally (4); $MgSO_4$ may have potential as an adjunct treatment with HT in term NE. Experimental data suggest increasing serum magnesium to twice baseline values is neuroprotective (5,6), however studies demonstrating efficacy have been confounded by co-existing accidental hypothermia (7) and those controlling core body temperature failed to demonstrate benefit (8). Clinical studies of $MgSO_4$ in term infants with NE mostly predate the implementation of hypothermia and were limited by small numbers, variable dosing regimens and inconsistent outcome measures (9). These trials implemented a daily bolus regimen of $MgSO_4$, resulting in significant peaks and troughs in serum magnesium, limiting exposure to supra-systemic magnesium concentrations. Such peaks and troughs in the serum magnesium have been associated with hypotension in a large animal model of NE (8).

$MgSO_4$ provides neuroprotection through the blockade of the glutamatergic NMDA receptors at post-synaptic neuronal membranes, preventing excessive calcium entry which would otherwise activate several injurious cellular pathways including catabolic enzyme induction and increased production of reactive oxygen species. Experimental data suggest $MgSO_4$ may also be anti-inflammatory; $MgSO_4$ modified the inflammatory cytokine response in pregnant rodents following exposure to a bacterial endotoxin, lipopolysaccharide (LPS) (10). Both $MgSO_4$ and HT are thought to reduce excitotoxicity and modulate the inflammation (11) and therefore it is plausible that these therapies may work synergistically. Indeed, combining $MgSO_4$ with HT in rodent models of NE has been shown to reduce infarct size compared to cooling alone (12).

We aimed to assess the neuroprotective benefit of a $MgSO_4$ bolus and constant infusion in combination with HT (Mg+HT) compared to hypothermia (HT) in a clinically-translational piglet model of NE. Primary outcome measures included amplitude-integrated electro-

encephalogram (aEEG) recovery after HI, magnetic resonance spectroscopy (MRS) biomarkers, and immunohistochemistry.

Methods

Ethics

All experimental and surgical procedures were performed in accordance with UK Home Office Guidelines [Animals (Scientific Procedures) Act, 1986] and the study complies with the ARRIVE guidelines.

Anesthesia and Surgical Preparation

Large White male piglets, aged <36h and weighing 1.7-2.1kg were anesthetized and surgically prepared as previously described (13). In brief, animals were sedated with intramuscular midazolam (0.2mg/kg) and anesthetized with isoflurane mixed with air (3% v/v during surgery, 1.5-2.5% during experimentation), remaining insentient throughout experimentation. Animals were mechanically ventilated via tracheostomy (SLE 2000 infant ventilator, Surrey, UK) and settings guided by arterial blood gas analysis (PaO_2 8-13kPa, pCO_2 4.5-6.5kPa). The common carotid arteries were surgically isolated and carefully encircled by inflatable carotid occluders (OC2A, In Vivo Metric). An umbilical arterial line was inserted for mean arterial blood pressure (MABP) and heart rate (HR) monitoring and umbilical venous line for infusions. Infusions included maintenance 10% dextrose at 60ml/kg/day (reduced to 40ml/kg/d post-insult), fentanyl 3-6mcg/kg/h and antibiotics (benzylpenicillin 50mg/kg/dose BD, gentamicin 5mg/kg/dose OD). The arterial line was infused with heparinized saline (0.5 IU/ml in 0.9% sodium chloride) at 0.3ml/hr.

Animals were nursed prone in a purpose-built MR compatible transport incubator. Intensive care was provided throughout the 48h experiment and complications (e.g. hypotension, seizures, hyperkalemia) were as per local neonatal guidelines. To maintain the MABP >40mmHg, 0.9% saline bolus (10ml/kg), dopamine (5–20µg/kg/min), dobutamine (5–20µg/kg/min), noradrenaline (0.1-1.5µg/kg/min) and adrenaline (0.1–1.5µg/kg/min) were used as required by a neonatologist.

Blood tests

Blood tests included pH, pCO_2, pO_2, bicarbonate (HCO_3), base excess (BE), lactate (Lac), glucose (Glu), urea and electrolytes (Abbot Laboratories, UK). Serum and cerebrospinal fluid (CSF) magnesium levels were frozen (-20^{o}C) and processed on completion of the study (Royal Veterinary College, Hawkshead, UK). Serum magnesium was taken at baseline, end of insult

(t=0) and 2, 3, 6, 12, 18, 24, 30, 36, 42 and 48h post-hypoxia ischemia (HI). CSF magnesium was measured at baseline and 48h.

Study Groups and Protocol

Block randomization of 15 animals was computer generated and sealed in opaque envelopes, opened on completion of HI. Animals were allocated to either $MgSO_4$ plus hypothermia (Mg+HT; n=8) or saline plus hypothermia (HT, n=7). Interventions were initiated 1h post-insult; whole body HT to 33.5^{o}C was maintained for 12h and $MgSO_4$ therapy was administered as 180mg/kg bolus plus 8mg/kg/h infusion over 48h **(Figure 1).**

Magnetic Resonance Spectroscopy

MRS was performed at 24h and 48h in a Philips clinical 3T MRI scanner. A 7x5cm elliptical transmit-receive MRS surface coil tuned to ^{31}P frequency (51.6MHz) was secured to the piglet's head and spectra were acquired at 1min intervals using a non-localised single pulse acquisition (repetition time 10s, 6 summed acquisitions per spectrum). Spectra were analyzed using the Advanced Method for Accurate, Robust and Efficient Spectral (AMARES) fitting of MRS data (14) as implemented in the jMRUI software. Nucleotide triphosphate (NTP) peaks were fitted as a doublet (α and γ) and triplet (β) structure with no assumption of the relative multiplet sizes. NTP is predominantly composed of adenosine triphosphate (ATP) and changes in this signal reflect changes in cerebral energetics. Measurements of inorganic phosphate (Pi), phosphocreatine (PCr), exchangeable phosphate pool (epp = *Pi + PCr + 2γ-NTP + β-NTP*) were acquired over the whole brain and peak area ratios calculated (Pi/epp, PCr/epp, NTP/epp).

^{1}H MRS spectra were acquired with a separate 6.5x5.5cm elliptical receive surface coil tuned to the ^{1}H resonance frequency. Metabolites were measured in a white matter voxel in the dorsal right subcortical region (8x8x15mm) and deep grey matter voxel (15x15x10mm) in the thalamus. The data was analyzed using jMRUI software and the lactate/N-acetylaspartate (Lac/NAA) peak area ratio calculated.

Amplitude integrated Electroencephalogram

A multichannel EEG (Nicolet) was acquired at baseline and continued for 48h post-insult. The aEEG score was classified as described by Hellstrom-Westas et al (1995) (15); isoelectric (0), continuous low voltage (1), burst suppression (2), discontinuous normal voltage (3) and continuous normal voltage (4). Scoring was performed hourly by two independent clinicians

(IL, KM) blinded to treatment allocation. aEEG scores were averaged in 6h time epochs and mean differences analyzed for significance.

Broadband Near-infrared Spectroscopy

An in-house developed broadband near-infrared spectroscopy (NIRS) optical measuring device (Mini-CYRIL, Cytochrome Research Instrument and Application System) provided real-time in vivo measurements of changes in cerebral concentration of oxyhaemoglobin (HbO_2), deoxyhaemoglobin (HHb) and oxidized cytochrome-c-oxidase (oxCCO). Changes in oxCCO during HI provided insight into the level of brain tissue metabolic suppression that is related to the degree of severity of the cerebral HI (16). The real-time and total fall in oxCCO during HI measured using the area under the curve below baseline (AUC oxCCO) was used as an indicator of insult severity (17).

Transient Hypoxia Ischemia

Animals were monitored for at least 1h to ensure haemodynamic stability, normal EEG and baseline broadband NIRS prior to HI. Carotid occluders were inflated and fraction of inspired oxygen (FiO_2) reduced simultaneously at the start of insult. FiO_2 was decreased to 6% and titrated to MABP, broadband NIRS and EEG. Oxygen delivery was liberalized in the event of MABP <27mmHg or 3-fold decrease in brain tissue oxidation of cytochrome-c-oxidase (oxCCO) as measured by broadband NIRS. Blood gas analysis was performed at 5min intervals during insult. Total duration of HI was determined by the duration of isoelectric EEG, hypotension (BP <30mmHg) in addition to total fall in oxCCO (AUC oxCCO) and reduction in FiO_2 (AUC FiO_2). Two experienced team members decided on the duration of insult using available information. At the end of insult the animal was resuscitated, occluders deflated and FiO_2 increased to 21%. The duration of HI was typically 18-22min.

Histology

Piglets were euthanized at 48h post-HI using a pentobarbital injection and brain fixed with an intra-cardiac injection with cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The brain was dissected out and fixation continued in 4% PFA for 7 days. The right cerebral hemisphere was dissected in 5mm coronal slices starting from the anterior optic chiasm and embedded in paraffin wax before being sectioned into 5µm slides. Two sections per piglet were stained (bregma 00 and −2.0) and 8 brain regions examined; cingulate cortex, sensorimotor cortex, hippocampus, internal capsule, periventricular white matter, caudate, putamen and thalamus **(Supplemental Figure S1).** All histological analysis was performed by an investigator blinded to treatment allocation.

To assess cell death and glial activation, sections were stained for nuclear DNA fragmentation using histochemistry with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL); the appearance of activated caspase 3 (CC3); glial fibrillary acidic protein (GFAP); and microglial ionised calcium-binding adaptor molecule (Iba1) immunoreactivity. Oligodendrocytes were stained with oligodendrocyte transcription factor (OLIG2) as a marker of myelination. For each animal, 2 sections placed 5mm apart were assessed for each stain.

For all histochemical and immunohistochemical stains, brain sections were dehydrated in xylene (3×10 min) and rehydrated in graded ethanol solutions (100–70%), followed by double-distilled water. For TUNEL, endogenous peroxidases were removed by pre-treating sections in 3% H_2O_2 in methanol, followed by a 15-min peptidase pre-digestion with 20µg/ml proteinase K (Promega) at 65°C. Sections were incubated at 37°C for 2h with TUNEL solution (Roche) containing biotinylated dUTP. TUNEL-positive cells were counted in 3 fields (x40 magnification; area 0.066mm^2) and averaged per region (cells/mm^2).

For activated caspase 3, Iba1 and OLIG2; pre-treatment with Ventana CC1 (950-124), equivalent to EDTA buffer was used. For GFAP, Protease 1 (0.38mg/mL alkaline protease enzyme activity) was used. Primary antibody incubation was performed with primary rabbit antibody against activated Caspase 3 (1:100, Cell Signalling 9661L) for 32min, Iba1 (1:250, WAKO 019-19741) for 4h, GFAP (1:1000, DAKO Z0334) for 32min and OLIG2 (1:100, Millipore AB9610) for 4h. Incubation with a secondary swine anti-rabbit immunoglobulin (DAKO E0343) was performed for a duration of 44min in activated caspase 3; 1h for Iba1 and OLIG2; and for 32min in GFAP staining.

The biotin residues were detected with avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories) and visualized with diaminobenzidine/H_2O_2 (Sigma), with $CoCl_2$ and $NiCl_2$ included to intensify TUNEL histochemistry. The sections were dehydrated in graded alcohol and xylene and mounted with Depex (VWR), or alternatively, mounted with Vectashield + 4',6-diamidino-2-phenylindole (DAPI) aqueous mounting media (Vector Labs), to facilitate total cell number counts during analysis of Iba1 and activated caspase 3.

Iba1 positive microglial cells were assigned a ramification index based on body and branch density using a 0.049x0.049mm square grid (x40 magnification) placed in three fields for each brain region. The microglial ramification index was calculated as B^2/C. B represents the average number of branches crossing the 3 horizontal and 3 vertical 0.049mm gridlines and

C the number of cell bodies within the grid. Activated caspase 3 immunoreactive cells were counted in 3 fields (x20 magnification; area 0.164mm^2) and averaged per region (counts/mm^2). Astroglial activation was quantified by measuring GFAP immunoreactivity optical luminosity values. The mean brightness values (two fields per region, x20 magnification) were deducted from the mean brightness of the blank slide (18). OLIG2 positive cells were counted in 3 fields (x40 magnification; area 0.066mm^2) and averaged per brain region (cells/mm^2).

Statistical Analysis

Statistical analysis was performed using Prism v6.0 for Mac, GraphPad Software, California USA. Parametric data was analyzed using student t-tests and non-parametric data with Mann-Whitney U tests. MRS, aEEG and immunohistochemistry were analyzed using ANOVA of the least mean squared difference. Histology was $\log_{10}$ transformed to normalize distribution for parametric statistical analysis.

Sample size calculation based on previous piglet studies (13) indicated that MRS biomarkers (Lac/NAA) over 48h varied by 1.0U between study groups (standard deviation 0.75) on a log scale. Using a significance threshold of 5% and 80% power, we determined 7 subjects were needed in each group.

Results

Physiological Measures

There were no significant differences in animal weight, baseline cardiovascular status (HR, MABP) and biochemistry (Na+, K+, urea, creatinine, glucose). Baseline blood gas was similar between groups with the exception of a slightly higher HCO_3 and BE in the Mg+HT group, although these values were within normal limits **(Table 1).**

The duration of HI, hypotension, isoelectric EEG, AUC oxCCO and AUC FiO_2 were similar between magnesium-treated and control animals. In addition, there was no significant difference in the end of insult blood gas; indicating the severity of HI was similar between groups **(Table 2).** One animal in each group had mild NE, defined *a priori* as aEEG recovery to at least discontinuous normal voltage within 1h post-insult.

Piglets were nursed in a thermoregulated mattress and normothermia was maintained throughout HI. Mean HR, MABP and temperature were similar between groups post-insult (0-1h), during induction of hypothermia (1-3h), hypothermia (3-13h), rewarming (13-23h) and normothermia (23-48h). Animals in the HT group had a slightly raised HR compared to Mg+HT

group, although this value was still within normal limits. Magnesium infusion was not associated with a significant increase in inotrope usage during HT or rewarming / normothermia **(Table 3).**

One piglet in the control group was euthanized early at 21h due to a rising lactate, and intractable hypotension (diagnosed with bowel perforation on post-mortem). Overall, there was no difference in survival between animals receiving Mg+HT and HT (8/8 vs 6/7, p=0.47).

Pharmacokinetics

Serum magnesium increased significantly by 1h following $MgSO_4$ bolus in the Mg+HT group (1.62 vs 0.72mmol/L, p=0.008); levels remained stable throughout infusion and were unaffected by HT **(Figure 2).** Interim analysis demonstrated a gradual fall in serum magnesium after 30h towards the lower end of the target range (1.40-2.00mmol/L). The infusion was increased from 8mg/kg/h (n=5) to 10mg/kg/h (n=3), however, overall serum magnesium levels did not change (1.51 vs 1.52mmol/L, p=0.5). CSF magnesium increased significantly from baseline to 48h post-infusion (1.21 vs 1.04mmol/L, p=0.008). Changes in CSF magnesium were relatively modest compared to serum; doubling serum magnesium resulted in a 16% rise in the CSF.

^{1}H and ^{31}P magnetic resonance spectroscopy

^{1}H and ^{31}P MRS were acquired in 14 out of 15 piglets at 24h and 48h. White matter and thalamic Lac/NAA were plotted on a logarithm scale; there was no significant difference in white matter Lac/NAA, thalamic Lac/NAA, Pi/epp, NTP/epp and PCr/epp observed between Mg+HT and HT groups at 24h and 48h **(Figure 3).** Exclusion of the 2 animals with mild HI did not alter these findings**.**

Amplitude-integrated Electroencephalogram (aEEG)

All animals had normal aEEG activity pre-insult with peak and baseline voltage greater than 10μV. Typically, aEEG became isoelectric (<5μv) within 3min of HI and did not recover when oxygen was liberalized during periods of excessive hypotension. Cerebral electrical activity remained severely suppressed, except 2 piglets demonstrated early aEEG recovery within 1h of HI (one in each group). There was a trend towards aEEG improvement after 36h in Mg+HT animals (p=0.09) which became significant when excluding two animals with mild injury (aEEG recovery within 1h post-HI) **(Figure 4).** Two animals in each group had seizures requiring treatment with 20mg/kg phenobarbitone (seizures were prolonged in one animal in each group).

Histology

Immunohistochemical staining with TUNEL, GFAP, Iba1, CC3 and OLIG2 was undertaken in all 15 piglets and quantitative analysis performed between treatment groups overall and by individual brain regions.

TUNEL

We observed a significant reduction in total brain TUNEL-positive cells in Mg+HT piglets compared to HT (21.1 vs 47.1 $\log_{10}$ count/mm^2, p=0.014) and a trend towards reduced cell death in the periventricular white matter (4.8 vs 22.3 $\log_{10}$ count/mm^2, p=0.094). There was no significant difference in cell death in the cingulate cortex, sensorimotor cortex, hippocampus, periventricular white matter, caudate and putamen **(Figure 5, Table 4).**

Cleaved Caspase 3

There was no difference in CC3 positive cells in Mg+HT piglets compared to HT (0.7 vs 0.5 $\log_{10}$ count/mm^2, p=0.29). There was, however, a significant increase in CC3-positive cells in the periventricular white matter in Mg+HT piglets compared to HT (1.4 vs 0.6 $\log_{10}$ count/mm^2) **(Figure 6a-d).**

Iba1

There was a trend towards increased ramification index (less microglial activation) for Iba1 positive cells in Mg+HT piglets compared to HT (1.6 vs 1.1, p=0.086). No significant differences were identified on analysis of individual brain regions **(Figure 6e-h).**

GFAP

There was no significant difference in overall GFAP optical luminosity in piglets receiving Mg+HT compared to HT (64.7 vs 57.1, p=0.11). On regional analysis, there was significantly higher luminosity in the cingulate cortex (59.2 vs 42.5, p=0.008) and caudate (49.7 vs 35.4, p=0.022) in piglets treated with Mg+HT compared to HT **(Figure 6i-l)**.

OLIG2

We observed an increase in the overall number of OLIG2 positive cells in Mg+HT piglets compared to HT (2.44 vs 2.05 $\log_{10}$ count/mm^2, p=0.002). Analysis of individual brain regions demonstrated a significant increase in OLIG2 positive cells in the hippocampus (2.49 vs 1.67 $\log_{10}$ count/mm^2, p=0.024) and thalamus (2.56 vs 1.6 $\log_{10}$ count/mm^2, p=0.004) in piglets treated with Mg+HT compared to HT **(Figure 6m-p).**

Discussion

This is the first large animal study comparing the neuroprotective efficacy of $MgSO_4$ with HT against HT alone. $MgSO_4$ administered as a bolus and infusion was well tolerated and provided a stable, raised serum magnesium concentration with significant rise in CSF 48h post-infusion. We observed an overall reduction in TUNEL cell death in animals treated with Mg+HT compared to HT, but on regional analysis there was no significant difference between groups. We observed an increase in surviving oligodendroglia in the hippocampus and thalamus. We saw more rapid aEEG recovery after 30h of treatment on post hoc analysis in the Mg+HT group (excluding the mild NE animals); but no improvement on MRS biomarkers at 24 or 48h post-HI.

Hypotension is a common side effect of $MgSO_4$ and previously observed following repeated boluses of $MgSO_4$ in normothermic piglets (8). Given the physiological effects of HT and cardiovascular depression following HI, hypotension is a significant concern with combination therapy. Rahman and colleagues reported a favourable safety profile in a clinical trial of $MgSO_4$ boluses with HT, but no cardiovascular parameters immediately following drug administration (19). Reassuringly, we did not observe significant hypotension or increased inotrope usage during HT, rewarming or normothermia. Serum magnesium pharmacokinetics remained stable during hypothermia and we did not observe toxic accumulation. Of note, a two-fold rise in serum magnesium resulted in a modest 16% increase in CSF concentration, consistent with data from adult neurosurgical studies (20). Magnesium is actively transported across the blood brain barrier through ATP-dependent ion exchangers and cation channels (21). Saturation of these ion channels may explain the limited rise in CSF concentration compared to serum.

Magnesium ions bind in a voltage dependent manner to the glutamatergic NMDA receptor channel and competitively antagonize calcium ion entry. The excessive release of excitatory neurotransmitters such as glutamate is a key mechanism of injury in the hours following HI and is a target for neuroprotective intervention. We observed a significant reduction in overall, but not regional, cell death in animals treated with Mg+HT, compared to HT. A trend to lower TUNEL-positive cells was seen in the periventricular white matter ($p=0.094$). Our data are consistent with those from an adult rodent study in which Mg+HT started 2h after global ischemia showed a small incremental improvement in neuroprotection with both mild and moderate HT, but the improvement was not significant (22). Such incremental improvement in cell survival would be difficult to detect in a reasonably sized clinical trial but may be

important in future pre-clinical studies aimed at optimizing outcomes with a cocktail of therapies.

Although excitotoxic mediated injury may affect all neuronal cells, the myelin-producing oligodendrocytes are particularly vulnerable (23). This may explain the increase in overall oligodendrocyte count (OLIG2 positive cells) and trend towards reduced cell death on TUNEL-positive cells in the white matter in animals receiving Mg+HT. Prolonged infusions of $MgSO_4$ have been associated with a reduction in the number of immature and mature oligodendrocytes in the intragyral and periventricular white matter in a preterm fetal sheep model (24). In our study we did not measure changes in immature or mature oligodendrocytes or myelination. In future studies it will important to assess whether add on treatment with $MgSO_4$ has differential effects on survival within the oligodendrocyte lineage and whether or not it can improve myelination.

Surprisingly, there was a non-significant increase in the CC3 cell count, particularly in the periventricular white matter in the Mg+HT compared to the HT group. Apoptotic pathways are sexually dimorphic (25) and in the male piglet (all piglets in our study were male), apoptosis mainly occurs via caspase-independent pathways (26), making CC3 a poor apoptotic marker in this model. CC3 alterations in our model appear to reflect caspase's non-apoptotic functions, including promoting microglial and lymphocyte function, cell differentiation and autophagy (27–29). We have previously observed a poor correlation between TUNEL-positive cell counts and CC3. MRS Lac/NAA used in both babies with NE and in our piglet model, shows a strong correlation with TUNEL-positive cells and weak correlation with CC3, suggesting TUNEL-positive cells are the most relevant marker for outcome in this model (30). Lastly, termination of the studies at 48h, may be insufficient time for apoptotic cell death to fully evolve and may partially explain the discrepant CC3 data.

aEEG is a useful clinical tool to stratify the severity of NE and recovery of electrical activity is associated with good clinical outcomes (31), although this may be delayed in infants undergoing HT (32). The trend towards aEEG recovery in the Mg+HT group is an indication of the potential incremental efficacy of $MgSO_4$ in animals, particularly in moderate to severe injuries. A similar number of piglets in each group had seizures detected on aEEG (2 in each group) and were treated with phenobarbitone. Unlike recent studies with $MgSO_4$ in fetal sheep (33), we did not observe any reduction in seizure burden in piglets who received Mg+HT. The possible brain injury related to phenobarbitone use itself (34) is likely to have been balanced in our study due to the equal incidence of seizures in each group

Cerebral Lac/NAA is a robust prognostic marker in the first 2 weeks after birth in babies with NE (35) and used as a surrogate measure of long-term outcomes in experimental trials of neuroprotection in NE (13,36). It was surprising that we did not observe significant improvement in MRS biomarkers in the Mg+HT treated piglets at 24 or 48h post-insult. It is possible that the additional protective effect of $MgSO_4$ was too small to be observed with MRS.

Magnesium has previously been reported to attenuate the inflammatory response after HI. $MgSO_4$ exposure significantly decreased IL6 and TNFα production in LPS-stimulated cord blood monocytes (37). $MgSO_4$ administered to pregnant rodents attenuated LPS-induced pro-inflammatory mediator expression (38) and improved offspring learning at 3 months (10). In our study, there was a trend towards increased overall ramification index in the Mg+HT group (p=0.086) compared to HT. Activation of brain-resident microglia comprises a set of highly conserved cellular responses to brain injury. Metabolic activation, migration, and loss of ramification associated with phagocytosis are rapid events that can occur within hours following injury. The partial preservation of the microglial ramification index is in keeping with the incremental neuroprotection seen with Mg+HT in this study. The ramification index provides a quantitative measure to rapidly assess total brain injury and has been seen previously to correlate negatively with TUNEL cell density and brain MRS measures (39).We observed an increase in astrogliosis in the cingulate cortex and caudate in the Mg+HT group which was unexpected.

This study was the first to utilise a $MgSO_4$ bolus and infusion to achieve a stable supra-systemic concentration of magnesium in a term-equivalent piglet model of NE. We have demonstrated baseline serum magnesium levels can be doubled safely and maintained over 48h. We felt initiating therapy by 1h is feasible in clinical practice and provides sufficient time to initiate resuscitation, gain intravenous access and commence magnesium therapy.

The main limitation of this study was the short duration (48h) after HI for injury to develop; the fate of microglia and apoptosis may therefore still be evolving. The main strengths of this study were its standardized HI insult, known timing of injury and clinically relevant outcomes measures. Although the study was powered *a priori* to detect a difference based on previous piglet studies, the minimal numbers used, keeping the 3Rs in mind, may explain the absence of a statistically significant reduction in MRS biomarkers. Animals were cooled for 12h, rather than 24h as in our previous studies (13); this was justified as we are modelling a situation where cooling is partially effective and the original study demonstrating efficacy of cooling in piglets showed efficacy with 12h HT (40). Indeed, the possible additional benefit of longer HT may abolish any incremental neuroprotection provided by Mg.

In conclusion, we demonstrated in a piglet model of NE treated with HT that a $MgSO_4$ bolus and infusion is a safe regimen to raise and maintain supra-physiological magnesium serum levels. Combined Mg+HT therapy significantly reduced overall neuronal cell death and increased numbers of myelin-producing oligodendrocytes. We did not however demonstrate significant improvement in aEEG score or MRS biomarkers, indicating that the overall neuroprotective benefit of combined Mg+HT is incremental and therefore unlikely to translate into substantive improvement in clinical trials. However, such an incremental effect with a good safety profile justifies further pre-clinical studies of $MgSO_4$ in combination with complementary therapies in the future.

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Figure Legends:

Figure 1. Study time-line. Following baseline data acquisition, piglets underwent cerebral HI. At the end of HI (time=0), piglets were randomized to (i) Therapeutic hypothermia (HT; 33.5°C) + Mg; or (ii) HT. Treatment was started 1h after HI. Piglets were maintained under intensive care for 48h following HI, prior to euthanasia. MRS was acquired at 24 and 48 h. aEEG and NIRS were acquired at baseline and in between MRS acquisitions.

Figure 2. Pharmacokinetics of $MgSO_4$ bolus and infusion. Serum magnesium concentrations (a) increased significantly from baseline and remained stable in the target range (1.4–2.0mmol/L). CSF magnesium (b) in Mg+HT animals was significantly higher at 48h than baseline and in HT piglets (error bars represent SEM, *p<0.05).

Figure 3. Magnetic resonance spectroscopy at 24 and 48h post-HI. All p values relate to the 48h acquisition. Least square mean difference with 95% CIs for: **a.** Lac/NAA (white matter), p= 0.142; **b.** Lac/NAA (thalamus), p=0.131; **c.** nucleotide triphosphate/exchangeable phosphate pool (NTP/epp), p=0.317; **d.** phosphocreatine/inorganic phosphate (PCr/Pi), p=0.152. Overlapping bars show evidence of no difference. Thal=thalamic; WM=white matter

Figure 4. Effects of Mg+HT compared to HT on aEEG scores over 48h post-HI in: (a) all piglets; and (b) piglets with moderate to severe HI (after exclusion of 2 piglets with mild injury defined as aEEG recovery within 1h after insult). Error bars represent 95% CI (*p<0.05). aEEG scored according to pattern classification (c).

Figure 5. Scatter plots with median and interquartile range for overall and regional brain TUNEL-positive cell counts. Comparing Mg+HT with HT, there was significant reduction in overall cell death (p=0.014). There was a non-significant trend towards reduced TUNEL-positive cells in the Mg+HT group in the periventricular white matter (p=0.094).

Figure 6. Scatter plots with median and interquartile range for overall and regional immunohistochemistry. Data are shown for CC3 (a-d), IBA1 ramification index (e-h), GFAP (i-l) and OLIG2 (m-p). For CC3, there was no difference between study groups overall (p=0.292), however in the periventricular white matter we observed increased CC3 in Mg+HT animals (p=0.007). For IBA1 ramification index, there was a trend towards an overall increased ramification index (less activation of microglia) (p=0.086) in Mg+HT animals with a similar trend in the hippocampus (p=0.071). For GFAP, there was no significant difference in

overall astrogliosis but localized increases in the cingulate cortex (p=0.008) and caudate (p=0.022) in Mg+HT animals. For OLIG2, there was a significant overall increase in oligodendrocytes in Mg+HT animals (p=0.002) with regional differences in the hippocampus (p=0.024) and thalamus (p=0.004).