Melatonin as an Adjunct to Therapeutic Hypothermia in a Piglet Model of Neonatal Encephalopathy: a Translational Study

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ABSTRACT

Therapeutic hypothermia is only partially protective for neonatal encephalopathy; there is an urgent need to develop treatments that augment cooling. Our objective was to assess safety, efficacy and pharmacokinetics of 5 and15mg/kg/24h melatonin (proprietary formulation) administered at 2h and 26h after hypoxia-ischemia (HI) with cooling in a piglet model. Following moderate cerebral HI, 30 piglets were eligible and randomized to: i) Hypothermia (33.5°C, 2-26h) and vehicle (HT+V;n=13); b) HT and 5mg/kg melatonin over 6h at 2h and 26h after HI (HT+Mel-5;n=4); c) HT and 15mg/kg melatonin over 6h at 2h and 26h after HI (HT+Mel-15;n=13). Intensive care was maintained for 48h; brain MRS was acquired and cell death (TUNEL) evaluated at 48h. Comparing HT+V with HT+Mel-5 and HT+Mel-15, there was no difference in blood pressure or inotropic support needed, brain Lactate/N Acetylaspartate at 24h and 48h was similar, ATP/phosphate pool was higher for HT+Mel-15 versus HT+V at 24h ($p=0.038$) but not 48h. A localized reduction in TUNEL positive cell death was observed in the sensorimotor cortex in the15mg/kg melatonin group (HT+Mel-15 versus HT+V; $p<0.003$) but not in the 5mg/kg melatonin group (HT+Mel-5 versus HT+V; $p=0.808$). Putative therapeutic melatonin levels were reached 8h after HI (10^4 increase from baseline; ~15-30mg/L). Mean $\pm$SD peak plasma melatonin levels after the first infusion were 0.0014$\pm$0.0012 mg/L in the HT+V group, 3.97$\pm$1.53 mg/L in the HT+Mel-5 group and 16.8$\pm$8.3 mg/L in the HT+Mel-15 group. Protection was dose dependent; 15mg/kg melatonin started 2h after HI, given over 6h, was well tolerated and augmented hypothermic protection in sensorimotor cortex. Earlier attainment of therapeutic plasma melatonin levels may optimize protection by targeting initial events of reperfusion injury. The time window for intervention with melatonin, as adjunct therapy with cooling, is likely to be narrow and should be considered in designing future clinical studies.

INTRODUCTION

Intrapartum-related insults at full term, such as hypoxia-ischemia (HI), are the 3rd leading cause of child death across the world (Liu et al., 2016). Each year, over 0.7 million affected newborns die and 1.15 million newborns develop acute disordered brain function known as neonatal encephalopathy (NE) (Kurinczuk et al., 2010). The incidence of NE in Western Europe and North America is around 1-3/1000 term births depending on the definitions used (Lee et al., 2013). Although improvements in care might prevent NE and neonatal death in some cases (NPEU, 2017), many cases cannot be prevented and therapies are limited. Therapeutic hypothermia (HT) initiated within 6h of birth improves outcome, yet despite this therapy 44-53% of infants with NE die or suffer moderate to severe disabilities including cerebral palsy, developmental delay, epilepsy and visual impairment (Edwards et al., 2010; Jacobs et al., 2011). Attempts to increase brain protection with deeper and longer cooling (Alonso-Alconada et al., 2015; Shankaran et al., 2014; Shankaran et al., 2017) suggest that current clinical cooling protocols are optimal and that other therapies which augment hypothermic neuroprotection in NE are needed (Robertson et al., 2012).

In a comparative review of potential neuroprotective agents that might augment HT for NE, melatonin (N-acetyl-5-methoxytryptamine) showed most promise in terms of efficacy and safety (Robertson et al., 2012). A strong body of evidence suggests that melatonin is neuroprotective for acute HI injury in the adult (Macleod et al., 2005) and neonatal brain (Husson et al., 2002) mediated by its anti-oxidant, anti-apoptotic and anti-inflammatory properties (Kilic et al., 2005; Luchetti et al., 2010). As HT is routine for term NE, it is important to assess if melatonin augments protection given by cooling and what plasma melatonin levels are safe and optimal. In a neonatal piglet model of perinatal asphyxia we previously showed that 30mg/kg/24h intravenous melatonin, started at 10mins after HI and infused over 6h with ethanol excipient was well tolerated, improved brain energy metabolism on magnetic resonance spectroscopy (MRS) and significantly reduced cell death in grey and white matter compared to cooling alone (Robertson et al., 2013); in that study, maximum peak melatonin levels (C_{max}) were 17-31 mg/L (74-134μM). A recent i*n vitro* study using organotypical hippocampal brain slices confirmed the synergy between cooling and melatonin for protection and concurred with these approximate levels (~15-30mg/L) from the previous *in vivo* piglet study for optimal neuroprotection with melatonin; in the *in vitro* study, melatonin reduced cell death in a concentration-dependent manner (1-100 uM) with a half maximal effective concentration (EC_{50}) of about 25 μM (equivalent to 5.8 mg/L) (Carloni et al., 2018).

Melatonin crosses the blood brain barrier and has an excellent safety profile with no known adverse effects (Buscemi et al., 2006; Jahnke et al., 1999). In small neonatal clinical studies, melatonin improved outcomes in sepsis (Gitto et al., 2001), prematurity (Gitto et al., 2004b), and perinatal asphyxia (Fulia et al., 2005). As melatonin is sparingly soluble in aqueous vehicles, solubility enhancers are needed, such as ethanol or other excipients, to obtain a solution with the desired

concentration. Ethanol, in particular, could have been a confounding factor in previous studies (Drury et al., 2014; Fulia et al., 2005; Welin et al., 2007; Yawno et al., 2017) since low doses of ethanol can have neuroprotective effects against ischemia/reperfusion injury (Su et al., 2017). Babies in the neonatal intensive care around the world are commonly exposed to potentially neurotoxic excipients, including ethanol; efforts are urgently needed to understand pharmacokinetics, long-term effects and safety of ethanol in excipients (Marek and Kraft, 2014) and potentially reduce this exposure (Whittaker et al., 2009).

In the present study, an ethanol-free, highly concentrated (5mg/ml) proprietary melatonin formulation was developed using excipients considered safe for use in neonates (Int. pat. appl. PCT/EP2018/056423); pilot pharmacokinetic studies showed that 15mg/kg of this melatonin formulation was well tolerated and achieved equivalent plasma levels to the 30mg/kg of the previously used ethanol-containing melatonin formulation in piglets (Robertson et al., 2013). We hypothesized that brain protection would be dependent on the melatonin dose and the area under the curve (AUC) melatonin within the therapeutic range. Our objective was to assess safety, efficacy and pharmacokinetics of an ethanol-free melatonin formulation in a newborn piglet model of brain injury, starting the infusion at a time compatible with the clinical practice (2h after HI). This model replicates neonatal intensive care with 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 HI (Azzopardi et al., 1989; Lorek et al., 1994), pattern of injury, neuropathology and cerebral MRS. The efficacy of melatonin protection over cooling alone was assessed using: (i) Cerebral MRS biomarkers, proton (^{1}H) MRS lactate/N acetyl aspartate (NAA) (Mitra et al., 2018) and phosphorus-31 (^{31}P) MRS for phosphocreatine/inorganic phosphate (PCr/Pi) and ATP/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 in 8 brain regions using TUNEL at 48h after HI.

MATERIAL AND METHODS

Sample size calculation

Our primary outcomes were cerebral lactate/NAA, ATP/epp and TUNEL positive cells. Previous work with our model with melatonin at 10 mins after HI has suggested that the change in lactate/NAA during 48h varied between cooling and cooling plus melatonin by 0.5U with a standard deviation of 0.3U (log scale). Assuming a similar effect magnitude with melatonin and similar variability at 48h and with 5% significance and 80% power, at least 9 subjects are needed in each group. A delay in the administration of melatonin from 10 mins to 2h was likely to reduce the effect size and so the group size was increased to n=13. Interim analysis was planned to assess futility of treatment after 20 cases.

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. Piglets were anesthetised and surgically prepared as described previously (Lorek et al., 1994). Criteria for entry into the study were: (i) normal aEEG /EEG at baseline after surgery; (ii) normal ^{1}H MRS at baseline with only detectable lactate seen on the MR spectrum; (iii) no pyrexia; (iv) recovery of the ^{31}P MRS NTP/epp to >70% baseline after cerebral HI to ensure standardization of the insult severity.

The study time-line is shown in **Figure 1.** Anaesthesia was induced by 4% v/v isoflurane through a facemask for around 5 minutes 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 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 8–13kPa and carbon dioxide ($PaCO_2$) at 4.5–6.5kPa, 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.

A 4 French double lumen umbilical venous catheter (Vygon, Swindon, UK) was inserted for infusion of maintenance fluids (10% dextrose, 60 ml/kg/day, reduced to 40ml/kg/day post insult), fentanyl (3µg/kg/h) and antibiotics (benzylpenicillin 50 mg/kg every 12h and gentamicin 2.5 mg/kg every 24h). A 2.5 French umbilical arterial catheter (Vygon) was inserted for continuous monitoring of heart rate and arterial blood pressure (MABP), and intermittent blood sampling was used to measure PaO_2,

$PaCO_2$, pH, electrolytes, glucose and lactate (Abbot Laboratories, UK). Arterial lines were maintained by infusing 0.9% saline solution (0.3mL/h) with 1 IU/mL heparin sodium.

Intensive care support for the animal throughout the 48h experiment and complications (e.g. hypotension, seizures, hyperkalemia) managed in accordance with local neonatal intensive care guidelines. All piglets received continuous physiological monitoring (SA instruments). 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), noradrenaline (0.1-1 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 (>7.0 mmol/l) was treated with 4 µg/kg salbutamol (10 µg/ml) over 10 min. Rectal temperature was maintained at 33.5^{o}C from 2-26h using a cooling water mattress (Tecotherm); rewarming occurred at 0.5^{o}C/hour. For the remainder of the study rectal temperature was maintained in the normothermic range (38.0-39.0^{o}C) using a radiant warmer during surgery and subsequently a heating water mattress (Tecotherm).

MR Methods

Animals were nursed prone in a stereotactic frame built within a MR compatible transport incubator which was purpose-built. Piglets were positioned within the bore of 9.4 Tesla Agilent MR scanner. ^{1}H and ^{31}P MRS data were acquired at baseline (for all piglets n=30) and at 24h (HT+V n=11, HT+Mel-5 n=4, HT+Mel-15 n=13) and 48h (HT+V n=8, HT+Mel-5 n=4, HT+Mel-15 n=12) after cerebral HI.

For ^{31}P MRS a 7cm x 5cm elliptical transmit-receive MRS surface coil tuned to the ^{31}P resonant frequency was positioned above the head. ^{31}P MRS was acquired with 1min 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. Nucleotide tri-phosphate (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.

For ^{1}H MRS, data were collected from voxels located in the dorsal right subcortical white matter at the centrum semiovale level (white matter voxel, 8x8x15mm) and in the deep grey matter centered on both lateral thalami (deep grey matter voxel, 15x15x10mm) using a combination of a 65 x 55 mm elliptical receive surface coil, a 150 mm diameter transmit volume coil and a LASER acquisition (TR = 5000ms, 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.

Cerebral hypoxia-ischemia (HI) and randomization

HI was induced inside the MR scanner by remotely inflating the vascular occluders around both common-carotid arteries, and simultaneously reducing FiO_2 to 6% (vol/vol). During HI the β-NTP peak height was continuously monitored using in-house Matlab (Mathworks) software. At the point at which β-NTP had fallen to 50% of its baseline value, FiO_2 was increased to 9%. When β-NTP fell to 40% baseline height the inspired oxygen fraction was titrated to keep the β-NTP peak height between 30% and 40% of its original height for a period of 12.5 minutes. At the end of HI the carotid arteries were de-occluded and the FiO_2 returned to 21%. Insult severity was calculated (Faulkner et al., 2011).

Following resuscitation, piglets were excluded if the β-NTP peak did not recover to >70% baseline within 1h of resuscitation. Eligible piglets were randomized (computer generated randomization) into 3 groups (HT+V, HT+Mel-5, HT+Mel-15) until interim analysis and then 2 groups (HT+V or HT+Mel-15) (Figure 1).

Melatonin delivery

Vehicle and proprietary melatonin vials (batch# P571-03 and batch# P571-07PL) were stored at room temperature. The proprietary melatonin formulation was administered intravenously; for the HT+Mel-5 group the infusion rate was 0.33ml/hr over 6h for a typical 2kg piglet; for the HT+Mel-15 group the infusion rate was 1ml/h over 6h for a typical 2kg piglet. The vehicle was administered at 1ml/h over 6h for a typical 2kg piglet.

aEEG

After surgical preparation, multichannel six-lead EEG monitoring (Nicolet, Care Fusion, Wisconsin, USA) was acquired at baseline and between MRS data acquisitions. Filtered amplitude-integrated EEG (aEEG) recordings were classified according to the pattern classification (Hellström-Westas et al., 1995). A score of 0 was flat trace; 1, continuous low voltage; 2, burst suppression; 3, discontinuous normal voltage; and 4, continuous normal voltage, at baseline and then every hour after HI. Scoring was performed at hourly intervals independently by two clinicians (IL, KM) blinded to the treatment allocation. Hourly aEEG scores were averaged in 6h time epochs and mean differences analysed for significance between study groups. Seizure activity was quantified. Electrographic seizures were treated with Phenobarbitone, initially 20mg/kg, followed by 10mg/kg for subsequent seizures, up to a maximum 40mg/kg.

Blood and CSF for Melatonin Pharmacokinetics

Blood was sampled at baseline and 1, 3, 6, 12, 18, 26, 27, 29, 32, 38, 44, 50h after the first melatonin infusion at 2h. Blood samples were collected in lithium/heparin tubes and centrifuged

immediately after collection. Plasma was separated and then stored at -20°C before analysis. When piglets were euthanized, in most cases a terminal cerebrospinal fluid (CSF) sample was collected and melatonin levels analyzed.

Melatonin Assay

Plasma melatonin levels were measured by Stockgrand Ltd following a modification of Fraser (Fraser et al., 1983). Samples were subjected to chloroform extraction, solvent was removed by evaporation under oxygen free nitrogen. Extracted melatonin was resuspended in assay buffer and further diluted to give concentrations within the standard curve range of 0 – 500 pg/ml. Pig plasma quality control samples and spiked pig plasma were included in each assay. Limit of detection of the assay was 3pg/ml. Quality control values were 27.8 ± 3.4 pg/ml coefficient of variation (CV) = 12.3%; 73.5 ± 7.9 pg/ml CV = 10.7%; 118.1 ± 12.7 pg/ml CV = 10.7%; 214.2 ± 27.6 pg/ml CV = 12.9%; 137.9 ± 21.1 ng/ml CV = 15.3%; 256.1 ± 38.9 ng/ml CV = 15.2%; 550.0 ± 37.2 ng/ml CV = 6.8%.

Brain histology

At 48 h after HI, piglets were euthanized with pentobarbital and the brain was fixed by cardiac perfusion with cold 4% paraformaldehyde, dissected out and post-fixed at 4ºC in 2% paraformaldehyde for 7 days. Coronal slices (5mm thick) of the right hemisphere, starting from anterior to the optic chiasma, were embedded in paraffin, sectioned to 8µm thickness and stained with hematoxylin and eosin to validate the bregma for analysis. For each animal, 2 sections (bregma 00 and -2.0) were stained and 8 different brain regions were examined (**Figure 2**).

TUNEL (8 brain regions)

To assess cell death, brain sections were stained for nuclear DNA fragmentation using histochemistry with transferase mediated biotinylated d-UTP nick end-labelling (TUNEL) as previously described(Robertson et al., 2013). Briefly, TUNEL sections were pre-treated in 3% hydrogen peroxide, subjected to a protease K pre-digestion (Promega, Southampton, UK) and incubated with TUNEL solution (Roche, Burgess Hill, UK). TUNEL was visualized using avidin-biotinylated horseradish complex (ABC, Vector Laboratories, Peterborough, UK) and diaminobenzidine/H_2O_2 (DAB, Sigma, Poole, UK) enhanced with $CoSO_4$ and $NiCl_2$. TUNEL sections were dehydrated and cover-slipped with DPX (VWR, Leighton Buzzard, UK). For each animal and brain region, TUNEL-positive nuclei were counted at two levels, and from 8 regions with 3 fields per region **(Figure 2)** by an investigator blind to the treatment group and the average converted into counts per mm^2.

GFAP (sensorimotor and cingulate cortex only)

The levels of astroglial activation was assessed via GFAP immunoreactivity (DAKO Z0334, dilution 1:1000). Mean ±SD of optical luminosity values were measured in 3 non-overlapping fields (x20 magnification) of the different brain regions of GFAP stained slides using Optimas 6.5 image

software. SD was subtracted from the mean of each field and the resulting value was subtracted from the value obtained from the surround glass.

Iba-1 (sensorimotor and cingulate cortex only)

The activation state of Iba-1 positive cells (Wako 019-19741, dilution 1:250) was assessed by scoring the process number, process complexity (primary, secondary and tertiary) and relative intensity of the soma staining from 0 to 4 (score 0, thin process with tertiary branches and able to visualise the cresyl violet counterstain through the Iba-1 of the soma; 1, tertiary processes but increased staining intensity of the soma; 2, reduced numbers of tertiary processes and thickening of secondary processes with intense enlarged soma; 3, loss of secondary processes and short thickened primary with intense soma; 4, no process and intensely stained soma). Scores were taken of 4 cells/FOV at x20 magnification (at set positions from the corners of the image) for a total of 24 cells/region/animal. Two investigators, blinded to treatment group, scored each image independently and their scores were averaged.

Cleaved caspase 3 (sensorimotor and cingulate cortex only)

The sections were processed for antigen retrieval (800-mW microwave irradiation in 0.1M citrate buffer, 10 min), followed by overnight incubation with primary rabbit antibody against activated caspase 3 (Cell signalling 9661L, dilution 1:100) and then 2h incubation with biotinylated secondary goat anti-rabbit immunoglobulin antibody (1:100 Jackson laboratory). Sections were dehydrated in graded alcohol and xylene and mounted with Depex (VWR)

Statistical Methods

MRS: All analyses were performed using the SAS JMP® v11.0.0 software. A statistical model was fitted to the ratios NTP/epp, PCr/Pi and Lac/NAA. An analysis of variance (ANOVA) model was fitted and the differences in the means on the log scale for the three treatment groups (HT+V, HT+Mel-5, HT+Mel-15) were estimated from the model at each time point with 95% confidence intervals (CI) for the differences. The differences in treatment group means are shown graphically using least square mean plots and standard error of the mean (SEM) error bars.

Amplitude intergrateded EEG (aEEG): Following the baseline scoring, scores were obtained hourly until 48h after HI. Each subject's scores were averaged over 0-6h, 7-12h, 13-18h, 19-24h, 25-30h, 31-36h, 37-42h and an ANOVA model fitted to the mean scores. The differences in the means between the treatment groups were estimated from the model at each of the timepoints with 95% CI for the differences.

TUNEL: An ANOVA model was fitted to the mean counts to give an estimate of the expected counts per mm^2. The overall difference between the means for the treatment groups, and treatment

differences across regions are presented with 95% CIs and graphically using 95% LSD error bars.

Iba-1, GFAP and CC3: An ANOVA model was fitted to the mean counts (using log transformed data for the Iba-1 and GFAP outcomes to satisfy normality assumptions), to investigate the effect of HT+Mel-5 and HT+Mel-15 compared to HT+V in the sensorimotor and cingulate cortex (areas where TUNEL positive cells were most affected by Mel-15).

3. RESULTS

Forty-eight piglets were studied. Five piglets were excluded prior to HI as they were in poor condition on baseline studies before HI (3 were pyrexial, one had high cerebral lactate/NAA and one had a flat EEG). Thirteen piglets were excluded just after cerebral HI due to no or poor NTP/epp recovery (<70% of baseline suggesting very severe insult). Thirty piglets were eligible for inclusion in the study and randomized after HI. There were 13 animals in HT+V group, 4 animals in the HT+Mel-5 group and 13 animals in the HT+Mel-15 group. The interim analysis (after 20 piglets) suggested futility in continuing the 5mg/kg melatonin group and this group was discontinued. The same randomization schedule was used as prior to the interim analysis, with the HT+Mel-5 group excluded.

Physiological data and insult severity

There were no significant intergroup differences between groups in bodyweight and postnatal age **(Table 1).** The HT+Mel-5 group showed higher blood lactate at baseline and at 12h after HI, $PaCO_2$ was increased compared to the other groups; the small group size may have influenced these values. No other differences were seen in any other parameter over the 48h study. There was no difference in the HI insult severity between groups **(Table 1).** There was no difference for volume replacement and inotrope use following HI between groups **(Table 2).**

9.4T MRS

The least squares mean plots and SEM bars for the NTP/epp, PCr/Pi and Lac/NAA (on log 10 scale) in thalamus and white matter are shown in **Figure 3.** Comparing HT+V with HT+Mel-5 and HT+Mel-15 groups, NTP/epp was higher in the HT+Mel-15 group at 24h (p=0.038) but not at 48h **(Figure 3A).** There was no difference in PCr/Pi at any time point **(Figure 3B)**. There was no difference between groups for Lac/NAA in the thalamus **(Figure 3C)** and white matter (**Figure 3D)** at 24 and 48h.

aEEG

There was no difference in the group mean hourly aEEG scores between groups at any timepoint **(Figure 4).** Three piglets had electrographic seizures, 2 in the HT+V group (one treated with phenobarbitone 20 mg/kg at 14h, one treated with phenobarbitone 20mg/kg and 10mg/kg at 9h and 12h respectively) and one in the HT+Mel-5 group (treated with phenobarbitone 20mg/kg and 10mg/kg at 13h and 17h respectively). No piglets in the HT+Mel-15 group had electrographic seizures.

TUNEL

The estimated mean TUNEL cells/mm^2 for the treatment groups are shown in **Table 3 and Figure 5.** TUNEL positive cells were counted at 2 brain levels **(Figure 2),** 8 regions, 3 fields per region. Over all brain regions, there was a trend towards lower TUNEL positive cells in the HT+Mel-15 versus the HT+V treatment group (p=0.069) with the estimated overall TUNEL positive cells/mm^2 42.1 points lower (95% CI -3.3 to 87.5) in the HT+Mel-15 group versus HT+V alone. In the sensorimotor cortex,

there was a significantly lower cell death in HT+Mel-15 versus HT+V with the estimated TUNEL positive cells per mm^2 195.1 points lower (95% CI 67.1 to 323.1;p=0.003). There was a trend towards lower cell death in the sensorimotor cortex in HT+Mel-15 versus HT+Mel-5 with the estimated TUNEL positive cells/mm^2 172.8 points lower (95% CI -8.3 to 353.8;p=0.061).

In co-labelling studies with TUNEL and GFAP in the sensorimotor cortex, we observed that the majority of TUNEL postive cells did not co-localize with GFAP, suggesting that the dying cells were not astrocytes. Further staining would be required to determine the exact identity of dying cells **(Supplementary Fig).**

Iba1, GFAP and Cleaved caspase 3 (CC3) in the sensorimotor and cingulate cortex

There were no differences between groups in the cingulate and sensorimotor cortex for Iba-1, GFAP and CC3 (**Figure 6**).

Pharmacokinetics

Basal mean ($\pm$SD) plasma endogenous melatonin levels were detectable in the HT+V group (C_{max} 0.0014 $\pm$0.0012 mg/L and AUC_{last} 0.015 $\pm$0.012 mg*h/L). Mean ($\pm$SD) plasma melatonin levels in the HT+Mel-5 group were ~10^3-fold higher (C_{max} 3.97 $\pm$1.53 mg/L and AUC_{last} 136 $\pm$61 mg*h/L). Mean ($\pm$SD) plasma melatonin levels in the HT+ Mel-15 group were ~10^4-fold higher (C_{max} 16.8 ± 8.3 mg/L and AUC_{last} 555 ± 266 mg*h/L). The HT+Mel-15 group but not the HT+Mel-5 group had plasma levels comparable with those associated with a neuroprotective effect in our previous study using the ethanol-based melatonin formulation (Robertson et al., 2013). PK modelling of the data from this current study suggested that an intravenous infusion of 18mg/kg melatonin started at 1h after HI and given more rapidly over 2h, would result in putative therapeutic plasma concentrations by 3h after HI (compared to 8h after HI in our current study) **(Figure 7).**

CSF melatonin levels at 48h in the HT+V group were low (ca.0.0001mg/L); in the HT+Mel-15 group CSF levels were 3.3 ± 1.3 mg/L. Importantly, the mean CSF/plasma melatonin concentration ratio (calculated at 48h) of 0.5 (range 0.3-1.0) in the HT+Mel-15 group demonstrated adequate CNS penetration.

DISCUSSION

Compared to cooling, the current standard therapy for babies with NE, there was a localized reduction in brain cell death in the sensorimotor cortex with the combination of cooling and a 15mg/kg proprietary intravenous melatonin formulation (PCT/EP2018/056423) given at 2h and 26h after cerebral HI in a piglet model of NE. In the sensorimotor cortex, there was a significantly lower cell death in HT+Mel-15 versus HT+V with the estimated TUNEL positive cells/mm^2 195.1 points lower (p=0.003). With this dose regimen in the HT+Mel-15 group, plasma melatonin levels were ~10^4 higher at the end of the infusion (8h after HI) than endogenous baseline levels, remaining in this range throughout the study. In the HT+Mel-5 group, plasma melatonin levels were 10^3 higher at the end of the infusion than endogenous baseline levels and did not reach previously proposed therapeutic range (Carloni et al., 2018; Robertson et al., 2013) at 8h or at any time after HI. The lower dose of melatonin (5mg/kg at 2 and 26h), although tested in a limited number of animals, did not show reduced TUNEL positive cells compared to cooling alone (estimated TUNEL positive cells/mm^2 22.3 points lower (p=0.808) in sensorimotor cortex), suggesting that higher plasma levels of melatonin are required for neuroprotection after cerebral HI, in accordance with *in vitro* data (Carloni et al., 2018; Skaper et al., 1998)

It is unclear why protection was only observed in the sensorimotor cortex with HT+Mel-15 at 2h and 26h combined with cooling from 2-26h. The sensorimotor cortex was the area of maximal injury in this study; melatonin targeted this area, reducing TUNEL positive cells but not affecting microglial activation or astrogliosis. Co-labelling studies suggested that dying cells were not astrocytes and therefore likely to be neurons. Such localized protection is unlikely to be related to vascular factors as melatonin is known to be highly brain penetrant (Paradies et al., 2010) and, indeed, was found in the CSF at concentrations comparable to plasma. Localized protection of the sensorimotor cortex combined with hypothermic protection, might lead to improved motor function which might be detectable at 18 months to 2 years in survivors of NE. In our previous study where melatonin was given at 10 mins after HI, protection was seen across both central deep grey and white matter areas with severe injury (Robertson et al., 2013). It is likely that the earlier administration of melatonin in this study was an important factor in the widespread protection (Robertson et al., 2013). Our data suggest that the time window for intervention with melatonin, as an adjunct therapy with cooling, is narrow, and should be taken into consideration in designing future clinical studies.

Melatonin is likely to be most effective early after HI through its diverse anti-oxidative mechanisms preventing free radical-induced oxidative damage to the electron transport chain and mitochondrial DNA (Reiter et al., 2016). Melatonin and its metabolites work as potent free radical scavengers, modulating the anti-oxidant capacity by enhancing anti-oxidant enzyme expression and activity (Galano et al., 2011; Galano et al., 2013). Melatonin also protects the integrity of the electron transport chain and increases the activity of complex I and IV so maintaining the mitochondrial energy

production. Melatonin thus maintains glutathione homeostasis in the mitochondria and increases ATP production (Leon et al., 2004). Melatonin subsequently prevents apoptosis both by directly stabilizing and preventing nitro-oxidative damage to membrane lipids, and by inhibiting pro-apoptotic proteins such as BAX; these actions prevent leakage of cytochrome c and propagation of the intrinsic apoptotic cascade. The newborn infant is at heightened risk for free radical production and injury from oxidative stress. Birth provides a hyperoxic challenge with transition from a low to high oxygen environment - this is amplified in the already hypoxic neonate who is then often resuscitated with supplemental oxygen (Gitto et al., 2013).

There was no difference in MRS at 48h; the MRS biomarkers measured in this study are known to correlate with injury severity after HI in the piglet (Lorek et al., 1994; Penrice et al., 1997) and outcome in infants with NE (Mitra et al., 2018). Higher ATP on ^{31}P MRS in infants with NE is associated with better long-term outcome in clinical studies (Azzopardi et al., 1989). We saw higher levels of ATP with HT+Mel-15 compared to HT+V at 24h but not 48h, this may relate to fewer subjects being studied at 48h. The ^{31}P MRS voxel includes brain tissue in deep grey and white matter, but does not include the cortical regions; this may account for the absence of a difference in ^{31}P MRS. High levels of thalamic lactate/NAA on MRS in neonates in the first month after birth are predictive of a poor 12-18 month neurodevelopmental outcome(Mitra et al., 2018); we saw no difference in Lac/NAA on white or grey matter MRS with HT+Mel-15 and HT+Mel-5 compared to HT+V. The localized protection seen in the sensorimotor cortex may account for the absence of a difference between groups as the ^{1}H MRS voxels sample the deep grey and white matter only. The amplitude integrated EEG (aEEG) background voltage and rate of aEEG recovery after HI are also predictive of neurodevelopmental outcome even in babies undergoing HT, with a positive predictive value of an abnormal background pattern of 0.82 at 48h (Csekő et al., 2013). aEEG reflects brain electrical activity across all cortical brain regions; we did not observe a difference between groups despite the protection seen in the sensorimotor cortex. It is likely that aEEG background activity was weighted by all cortical areas and not able to detect localized sensorimotor cortex protection.

In this current study, cooling from 2-26h with melatonin (5mg/kg or 15mg/kg intravenous infusions over 6h) started 2h after HI and repeated at 26h after HI, was well tolerated and did not lead to any acute or persisting changes in cardiovascular status or mean blood pressure. This is consistent with previous reports of physiological stability of melatonin in newborns even in high doses (Fulia et al., 2005; Gitto et al., 2001; Gitto et al., 2004a). No study of antenatal or postnatal melatonin treatment has shown any serious side effects (Buscemi et al., 2006; Jahnke et al., 1999). Fulia *et al* (Fulia et al., 2005) treated asphyxiated term neonates within 6h of birth with enteral melatonin at a total dose of 27mg/kg over 14h which resulted in a reduction in blood markers of lipid peroxidation and nitric oxide (NO) synthesis. Aly et al (Aly et al., 2015) administered enteral melatonin as an adjunct to HT in term NE with a total 50mg/kg dose over 5 days, commenced within 6h. Infants treated with

melatonin had reduced plasma levels of NO and plasma superoxide dismutase after 5 days; there was also reduced white matter injury on MR, reduced seizure activity on EEG at 2 weeks and increased survival free of disability at 6 months compared with infants treated with HT. Both these studies used the enteral route for melatonin, either dissolved in ethanol (Fulia et al., 2005) or water (Aly et al., 2015). Aly et al demonstrated only a modest doubling of plasma melatonin levels from baseline to day 5 with 10mg/kg/day enteral melatonin (21±2.4 to 42.7±5.1 pg/ml ($p<0.001$), suggesting that the intravenous route of administration is much more effective in elevating plasma concentrations. Interestingly the HT group (without enteral melatonin) also showed an increase in in plasma melatonin from baseline to day 5 (20.6±2.5 to 32.1±3.5 pg/ml, $p<0.001$), confirming an endogenous melatonin response previously seen in brain injury (Seifman et al., 2008).

In our study, in the HT+Mel-15 group, plasma melatonin levels increased from 0.0014 ± 0.0012 mg/L at baseline to 16.8 ± 8.3 mg/L at 8h after HI - an increase of ~10^4. *In vitro* studies using organotypical hippocampal brain slices and ischemia simulated by oxygen and glucose deprivation, suggest that melatonin reduces cell death in a concentration-dependent manner (1-100 uM) with an EC50 of about 25 µM (equivalent to 5.8 mg/L) (Carloni et al., 2018). Extrapolating from this study (Carloni et al., 2018) and our previous piglet study (Robertson et al., 2013), it is likely that plasma melatonin levels of 8-32mg/L are necessary for maximal neuroprotection after acute HI in the term brain and that the earlier attainment of therapeutic levels after HI maximize protection. In a fetal sheep model of preterm HI, a continuous melatonin infusion from 2-26h (0.2mg intravenous melatonin bolus followed by 0.1mg/h for the next 24 h with ethanol excipient) led to a 70-fold increase in plasma levels at 10h compared to baseline (pre-melatonin infusion values; 10.1$\pm$4.4 pg/ml, peak levels 1717$\pm$46 pg/ml) (Yawno et al., 2017). Although white matter protection was observed, the influence of ethanol was unclear (Yawno et al., 2017). Recently a term asphyxia sheep model with 60mg melatonin started at 30 mins after HI either intravenously or as a transdermal patch was protective (Aridas et al., 2018). The intravenous formulation used ethanol as an excipient; melatonin levels increased 600 fold with a 60mg dose given over 24h. Although plasma levels were considerably lower than the putative target therapeutic levels in our piglet study, protection was observed. The contribution of ethanol to protection in the intravenous group is unclear however (Aridas et al., 2018).

It is possible that the ethanol, previously used at sub-toxic doses as an excipient to dissolve melatonin, influenced brain protection in our perinatal asphyxia piglet model (Robertson et al., 2013). A study in fetal sheep with low-dose melatonin (0.1 mg/kg over 6h) showed partial protection confounded by the ethanol used to dissolve melatonin (Drury et al., 2014). Other studies (Miller et al., 2005; Welin et al., 2007; Yawno et al., 2017) have not examined specific effects of ethanol when used as an excipient with melatonin. This is an important issue as neuroprotection has been observed in experimental adult models of stroke (Aronowski et al., 2003; Wang et al., 2012) with ethanol doses of 1.0 to 1.5 g/kg, which produce blood levels (89 mg/dL) within the legally intoxicated range (80–100 g/dL), within 4h

of middle cerebral artery occlusion (Wang et al., 2012). Possible mechanisms of protection from acute low dose ethanol include increased HIF-1α expression, however given the unknown risks associated with ethanol exposure in babies (Boschen and Klintsova, 2017; Klintsova et al., 2007), it is likely that a melatonin formulation free of ethanol will be needed to comply with safety regulations for clinical trials.

There are some limitations of our study. The delay of 8h for the attainment of C_{max} due to the slow infusion protocol used in this study may have reduced the neuroprotective effect of melatonin; the importance of early intervention has been seen with HT (Thoresen et al., 2013) and in the recent early phase clinical trial of xenon-augmented hypothermia in NE where xenon given at ~10h after birth had no benefit (Azzopardi et al., 2016). Pharmacokinetic modelling suggests that 18mg/kg melatonin started at 1h after HI and given over 2h would achieve levels within the therapeutic range by 3h; this protocol needs to be assessed in future safety and efficacy studies. The 15mg/kg dose was the primary target in this study, based on previous studies (Robertson et al., 2013); fewer piglets were studied in the 5mg/kg group than the 15mg/kg group and it is possible that a small effect was missed. Our outcome measures (aEEG, MRS and TUNEL) are surrogate measures of neurodevelopmental outcome and no neurodevelopmental assessment was possible in our subjects. Although the groups were similar at baseline, the HT+Mel-5 group showed higher blood lactate at baseline and $PaCO_2$ was increased at 12h compared to the other groups; the small group size may have influenced these values. There are some important strengths of this study: (i) a standard piglet hypothermia protocol was used which is known to be protective in previous studies; (ii) the comparison of doses was based on melatonin plasma levels and not just on a nominal dose; (iii) a dose response was evaluated; (iv) the insult severity was the same between groups and those piglets with severe or mild injury were excluded based on pre-defined criteria (<70% NTP/epp recovery) on ^{31}P MRS; (iv) there was delay of 2h in starting HT and melatonin reflecting the time of intervention due to the need of clinical assessment of a baby with NE (v) to avoid potential confounding effects, ethanol, an excipient frequently utilized to dissolve melatonin, was not utilized in this study.

We saw no effect of melatonin on brain microglial activation in this study. Melatonin is known to enhance the immune response through augmenting T-helper response (Brzezinski, 1997), reducing production of pro-inflammatory cytokines and reducing polymorphonuclear leukocyte recruitment (Gitto et al., 2013; Lowes et al., 2013). Melatonin can also effectively modulate phagocytosis and expression of proinflammatory mediators in activated microglia (Lowes et al., 2013). Earlier attainment of therapeutic melatonin levels may be important for this effect; we previously observed attenuation of microglial activation and modulated microglial phenotype with melatonin started 10 mins after HI (Robertson et al., 2013). Similarly, we saw no effect of melatonin on cleaved caspase 3 (CC3), usually associated with apoptosis. We have previously observed this disconnect between TUNEL positive cells and CC3 in our 48h model, which may not allow sufficient time for apoptotic cell death to fully

evolve. It is also possible that cell death occurred by processes independent of caspase 3, termed caspase independent cell death (CICD), which has been described in various conditions including ischemic stroke (Cho and Toledo-Pereyra, 2008). CICD processes are not dependent on functional caspases for completion and might therefore serve as an alternative death pathway in the face of cellular energy depletion that precludes caspase activation. In our study, no effect on astrogliosis was seen, a finding in accordance with our previous study (Robertson et al., 2013).

In summary, compared to cooling alone, brain cell death was reduced in the sensorimotor cortex with cooling and 15mg/kg proprietary intravenous melatonin formulation given 2h and 26h after cerebral HI in a piglet model of NE. Plasma melatonin levels increased ~10^4 by 8h after HI with 15mg/kg intravenous proprietary melatonin formulation, reaching the putative target therapeutic range at 8h and remaining at this level for 48h. A lower dose of melatonin (5mg/kg at 2 and 26h) did not reduce TUNEL positive cells in the sensorimotor cortex compared to cooling alone; melatonin levels did not reach the putative therapeutic range with this dose. These data suggest that brain protection after cerebral HI is dependent on plasma melatonin levels. Melatonin was well tolerated and did not lead to any detectable physiological or biochemical change. The earlier attainment of target plasma melatonin levels may target reperfusion-related oxidative stress, mitochondrial dysfunction and microglia activation and may further augment neuroprotection. For future studies, based on PK modelling from this study, a dose of 18mg/kg melatonin given intravenously at 1h after HI with a faster infusion rate (over 2h) is likely to reach putative therapeutic concentrations by 2-3h (compared to 8h in our current study). Further pre-clinical studies are needed to define the safety and optimal dose regimen of melatonin as an adjunct to HT in NE.

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Figure Legends

Figure 1.

Study time line. Following baseline data acquisition and screening for health, piglets underwent cerebral hypoxia-ischemia. Following hypoxia-ischemia and cerebral NTP recovery > 70%, piglets were randomized to (i) Hypothermia (33.5^{o}C from 2-26h) + Vehicle (HT+V); (ii) HT+ 5mg/kg melatonin (Mel-5) at 2h and 26h and infused over 6h; (iii) HT +15mg/kg melatonin (Mel-15) at 2h and 26h and infused over 6h. Piglets were maintained under intensive care for 48h following HI. MRS was acquired at baseline, during HI and 60 mins after resuscitation, at 24 and 48h. aEEG was acquired at baseline and between MRS studies.

Figure 2.

The two brain levels (A and B) sampled are shown as well as the 8 brain regions assessed for immunohistochemistry (3 fields per region, 2 levels). The red squares represent those areas sampled by MRS and the black squares those regions not sampled by MRS.

Figure 3.

Magnetic resonance spectroscopy of the brain at baseline, 24h and 48h after hypoxia ischemia. All groups underwent therapeutic hypothermia (HT) from 2-26h. Least square mean plots with standard error of the mean (SEM) error bars are shown for **(Figure 3A)** NTP/epp and **(Figure 3B)** PCr/Pi in whole forebrain and thalamic **(Figure 3C)** and white matter **(Figure 3D)** Lac/NAA for vehicle, Mel-5 and Mel-15 groups. NTP/epp was higher in the HT+Mel-15 group at 24h (p=0.038) but not at 48h. There were no other differences between groups. epp=exchangeable phosphate pool; Lac=lactate; NAA=N-acetylaspartate; WM=white matter; HI=hypoxia–ischemia.

Figure 4.

Amplitude-integrated electroencephalogram (aEEG). Scores were obtained hourly until 48h and averaged over 6 hourly periods. An ANOVA model was fitted and the differences in the means between the treatment groups estimated with 95% CIs for the differences There was no difference in the group mean hourly aEEG scores between groups. Representative aEEG traces for each score are shown in the lower panel.

Figure 5

Mean TUNEL counts for HT+V, HT+Mel-5 and HT+Mel-15. **Fig 5A** shows TUNEL counts across 8 different brain regions. There is a significant reduction in TUNEL positive cells in the HT+Mel-15 group compared to HT+V in the sensorimotor cortex (p=0.003). Representative TUNEL sections of the sensorimotor cortex are shown in **Fig 5B-D.** cCTX = cingulate cortex; sCTX = sensorimotor

cortex; Hip = hippocampus; pvWM = periventricular white matter; IC = internal capsule; Caud = caudate nucleus; PTMN = putamen; THAL = thalamus.

Figure 6

Immunohistochemistry in the sensorimotor and cingulate cortex in HT+V, HT+Mel-5 and HT+Mel-15 groups. The top panel shows cleaved caspase 3 (CC3), middle panel IBA-1 staining and lower panel GFAP. There was no difference in any of the immunohistochemical markers between groups.

Figure 7

Mean plasma concentrations (± SD) of the HT+Mel-5 (red line) and HT+Mel-15 groups (blue line) compared with the historical data from Robertson et al., 2013 (green line); plasma concentrations of the HT+V group (< 0.125 mg/L) could not be plotted due to the Y axis scale used. PK modelling for a proposed dose of 18mg/kg infused over 2h and started at 1h after HI is shown by dotted orange line (this dose and infusion rate was modelled on the current PK data and not tested in this current study). All concentrations are plotted considering the end of HI as t=0.

Supplementary Figure

Co-localization of TUNEL+ cell death (A, D, G) and astroglial cell lineage (GFAP) marker (B, E, H) in the sensorimotor cortex. TUNEL (red) , GFAP (green), DAPI (blue) in vehicle (A-C), low dose (D-F) and high dose (G-I). In the majority of cells under all three conditions there was no co-localization suggesting the dying cells were not astrocytes.

TABLES

Table 1. Baseline group data and physiological variables throughout the study

Parameters	Vehicle Mean (SD)	5mg/kg/24h Mean (SD)	15 mg/kg/24h Mean (SD)	P value
Body weight (g)	1916.15 (193.46)	1862.50 (110.87)	1977.69 (120.63)	.378
Duration of HI (min)	26.31 (4.03)	24.25 (2.22)	26.46 (4.16)	.602
Insult severity (10−2) measured from acute energy depletion (AED)	0.08 (0.04)	0.10 (0.02)	0.08 (0.04)	.508
Heart rate (min−1)				
Baseline	173.63 (15.24)	171.92 (8.82)	178.45 (20.44)	.706
End of insult (time 0)	186.91 (20.27)	180.31 (24.28)	191.60 (32.72)	.751
1–2 h after time 0	177.77 (20.38)	182.88 (35.65)	173.54 (18.09)	.732
2–26 h after time 0	151.85 (22.41)	160.76 (19.85)	153.42 (16.81)	.735
26–48 h after time 0	177.07 (23.2)	155.81 (13.2)	169.64 (12.27)	.132
Mean arterial blood pressure (mm Hg)				
Baseline	47.89 (5.73)	41.58 (1.23)	47.10 (5.54)	.129
End of insult (time 0)	43.76 (6.67)	38.06 (5.28)	43.17 (6.61)	.310
1–2 h after time 0	42.73 (7.05)	43.06 (8.82)	44.19 (6.36)	.865
2–26 h after time 0	45.99 (3.77)	42.99 (3.61)	48.23 (6.63)	.205
26–48 h after time 0	50.51 (6.52)	52.93 (7.53)	51.69 (6.48)	.799
Rectal temperature (°C)				
Baseline	38.23 (0.56)	38.46 (0.2)	38.29 (0.31)	.649
End of insult (time 0)	37.91 (0.41)	38.10 (0.3)	38.17 (0.56)	.399
1–2 h after time 0	37.98 (0.98)	38.29 (0.39)	38.10 (0.79)	.802
2–26 h after time 0	33.71 (0.11)	33.89 (0.18)	33.73 (0.25)	.250
26–48 h after time 0	36.59 (1.01)	37.12 (0.14)	37.05 (0.39)	.219
PaO2 (kPa)				
Baseline	16.59 (11.02)	16.05 (5.12)	16.73 (5.7)	.990
End of insult (time 0)	11.26 (2.76)	10.93 (2.19)	10.85 (2.27)	.910
12 h after time 0	12.35 (2.93)	10.28 (1.03)	10.66 (3.68)	.317
24 h after time 0	12.28 (2.11)	9.78 (2.74)	14.02 (4.75)	.127
48 h after time 0	13.40 (2.61)	13.00 (0)	17.47 (9.09)	.494
PaCO2 (kPa)				
Baseline	6.55 (3.73)	5.17 (1.13)	5.72 (1.8)	.597
End of insult (time 0)	5.04 (1.03)	5.54 (1.28)	4.96 (1.51)	.739
12 h after time 0	4.61 (1.42)	6.60 (1.17)	4.84 (1.05)	**.029**
24 h after time 0	4.67 (1)	5.08 (1.09)	4.32 (0.51)	.239
48 h after time 0	5.28 (0.62)	4.94 (0)	5.17 (1.18)	.944
Blood pH				
Baseline	7.41 (0.17)	7.46 (0.02)	7.47 (0.15)	.629
End of insult (time 0)	7.33 (0.08)	7.34 (0.17)	7.34 (0.13)	.983
12 h after time 0	7.51 (0.12)	7.41 (0.04)	7.53 (0.11)	.187

24 h after time 0	7.49	(0.08)	7.50	(0.11)	7.54	(0.07)	.406
48 h after time 0	7.42	(0.04)	7.41	(0)	7.45	(0.09)	.714
Base excess (mmol/l)							
Baseline	4.08	(4.42)	3.50	(5.51)	5.92	(3.48)	.420
End of insult (time 0)	-6.00	(5.26)	-4.00	(6.06)	-6.38	(3.75)	.692
12 h after time 0	4.54	(5.86)	5.75	(2.5)	7.31	(3.86)	.352
24 h after time 0	3.33	(4.91)	6.25	(3.2)	5.08	(3.62)	.389
48 h after time 0	1.29	(1.94)	-1.00	(0)	3.00	(3.79)	.354
Lactate (mmol/l)							
Baseline	3.59	(2.26)	7.55	(2.04)	4.59	(2.16)	**.013**
End of insult (time 0)	9.19	(2.36)	10.09	(1.22)	9.51	(2.52)	.803
12 h after time 0	3.08	(2.09)	2.03	(0.41)	2.70	(1.13)	.485
24 h after time 0	2.30	(0.83)	1.71	(0.46)	2.27	(0.98)	.474
48 h after time 0	1.48	(0.71)	2.19	(0)	1.23	(0.29)	.105
Glucose (mmol/l)							
Baseline	5.88	(0.98)	6.58	(1.46)	6.02	(1.12)	.541
End of insult (time 0)	9.20	(2.29)	9.33	(3.11)	8.55	(1.61)	.678
12 h after time 0	8.65	(3.52)	10.1	(4.1)	8.50	(2.24)	.646
24 h after time 0	10.83	(4.17)	10.8	(2.59)	9.89	(3.97)	.821
48 h after time 0	5.76	(1.48)	4.9	(0)	5.63	(1.14)	.810

Table 1. Time zero was set at the time of reperfusion/resuscitation. Mean $\pm$ standard deviation (SD) values are presented for the three groups; (i) HT+V (n=13); (ii) HT+Mel-5 (n=4); (iii) HT+Mel-15 (n=13). Apart from a higher lactate at baseline and higher CO_2 at 12h in the HT+MEL-5 group, analysis using Mann Whitney test indicated that there was no evidence of a difference between the two groups for any of the outcomes at any of the time-points. Insult severity was estimated by calculating the time integral of the change in NTP/epp during HI and the first 60 min of resuscitation.

Infusions	HT+V Mean (SD)		HT+Mel-5 Mean (SD)		HT+Mel-15 Mean (SD)		P value
Dopamine (µg/kg/min)	10.869	(5.314)	11.964	(5.404)	13.500	(3.255)	0.647
Dobutamine (µg/kg/min)	5.24	(-4.905)	3.405	(5.304)	7.811	(1.822)	0.277
Noradrenaline(ng/kg/min)	9.686	(17.142)	7.731	(17.122)	2.171	(4.342)	0.716
Adrenaline (ng/kg/min)	0.753	(2.827)	0	(0)	0.000	(0.000)	0.536
Saline Bolus (ml/kg)	0.166	(0.123)	0.138	(0.068)	0.192	(0.077)	0.565

Table 2. Average total volume replacement and inotrope infusion for the piglets in the HT+V, HT+Mel-5 and HT+Mel-15 groups during the 48h after HI.

Area	Comparison	Difference in Mean TUNEL Count	SE of Difference	Lower 95% C.I. for Difference	Upper 95% C.I. for Difference	*p*-value
Sensorimotor cortex	**HT+V v. HT+Mel-15**	**195.1**	**64.9**	**67.1**	**323.1**	**0.003**
	HT+V v. HT+Mel-5	22.3	91.8	-158.7	203.4	0.808
	HT+Mel-5 v. HT+Mel-15	172.8	91.8	-8.3	353.8	**0.061**
OVERALL	**HT+V v. HT+Mel-15**	**42.1**	**23.0**	**-3.3**	**87.5**	**0.069**
	HT+V v. HT+Mel-5	**-0.9**	**32.5**	**-65.0**	**63.2**	**0.977**
	HT+Mel-5 v. HT+Mel-15	**43.0**	**32.5**	**-21.0**	**107.0**	**0.187**

Table 3. Differences between HT+V, HT+Mel-5 and HT+Mel-15 group TUNEL counts for the sensorimotor cortex and overall (8 brain regions). The other brain regions did not show a significant difference in TUNEL counts.