Melatonin as an adjunct to therapeutic hypothermia in a piglet model of neonatal encephalopathy: A translational study

Nicola J. Robertson[a,b,*], Kathryn Martinello[a], Ingran Lingam[a], Adnan Avdic-Belltheus[a], Christopher Meehan[a], Daniel Alonso-Alconada[c], Sara Ragab[a], Alan Bainbridge[d], Magdalena Sokolska[d], Mohamed Tachrount[e], Benita Middleton[e], David Price[d], Mariya Hristova[a], Xavier Golay[f], Annamaria Soliani Raschini[g], Giancarlo Aquino[g], Nicola Pelizzi[g], Fabrizio Facchinetti[g]

[a] University College London, London WC1E 6HX, UK
[b] Division of Neonatology, Department of Pediatrics, Sidra Medicine, Doha, Qatar
[c] University of the Basque Country, Leioa, Spain
[d] University College London Hospitals NHS Trust, UK
[e] Chronobiology Group, Faculty of Health & Medical Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK
[f] Institute of Neurology, Queen Square, University College London, London, UK
[g] Chiesi Farmaceutici S.p.A., Parma, Italy

A B S T R A C T

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 and 15 mg/kg/24 h melatonin (proprietary formulation) administered at 2 h and 26 h 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, 2–26 h) and vehicle (HT + V;n = 13); b) HT and 5 mg/kg melatonin over 6 h at 2 h and 26 h after HI (HT + Mel-5;n = 4); c) HT and 15 mg/kg melatonin over 6 h at 2 h and 26 h after HI (HT + Mel-15;n = 13). Intensive care was maintained for 48 h; brain MRS was acquired and cell death (TUNEL) evaluated at 48 h. 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 24 h and 48 h was similar, ATP/phosphate pool was higher for HT + Mel-15 versus HT + V at 24 h (p = 0.038) but not 48 h. A localized reduction in TUNEL positive cell death was observed in the sensorimotor cortex in the 15 mg/kg melatonin group (HT + Mel-15 versus HT + V; p < 0.003) but not in the 5 mg/kg melatonin group (HT + Mel-5 versus HT + V; p = 0.808). Putative therapeutic melatonin levels were reached 8 h after HI (10^4 increase from baseline; ~15–30 mg/l). Mean ± SD peak plasma melatonin levels after the first infusion were 0.0014 ± 0.0012 mg/l in the HT + V group, 3.97 ± 1.53 mg/l in the HT + Mel-5 group and 16.8 ± 8.3 mg/l in the HT + Mel-15 group. Protection was dose dependent; 15 mg/kg melatonin started 2 h after HI, given over 6 h, 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.

1. 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

6 h 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 neuro-protective 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 30 mg/kg/24 h in-travenous melatonin, started at 10 min after HI and infused over 6 h with ethanol excipient was well tolerated, improved brain energy me-tabolism 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 (74–134 μM). A recent in vitro study using organotypical hippocampal brain slices confirmed the synergy between cooling and melatonin for protection and concurred with these ap-proximate levels (~15–30 mg/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 con-centration. 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 neuro-protective 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 po-tentially reduce this exposure (Whittaker et al., 2009).

In the present study, an ethanol-free, highly concentrated (5 mg/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 15 mg/kg of this melatonin formulation was well tolerated and achieved equivalent plasma levels to the 30 mg/kg of the previously used ethanol-containing melatonin formulation in piglets (Robertson et al., 2013). We hy-pothesized that brain protection would be dependent on the melatonin dose and the area under the curve (AUC) melatonin within the ther-apeutic range. Our objective was to assess safety, efficacy and phar-macokinetics 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 (2 h after HI). This model replicates neonatal intensive care with monitoring and control of physiological and meta-bolic 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, neuro-pathology 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 48 h after HI.

2. Material and methods

2.1. 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 min after HI has suggested that the change in lactate/NAA during 48 h varied between cooling and cooling plus melatonin by 0.5 U with a standard deviation of 0.3 U (log scale). Assuming a similar effect magnitude with melatonin and similar variability at 48 h 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 min to 2 h 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.

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. Piglets were anesthetised and surgically pre-pared 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 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 mon-itoring (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 8–13 kPa and carbon dioxide ($PaCO_2$) at 4.5–6.5 kPa, al-lowing for temperature and fraction of inspired oxygen (FiO_2) correc-tion 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 40 ml/kg/day post insult), fentanyl (3 μg/kg/h) and antibiotics (benzylpenicillin 50 mg/kg every 12 h and gentamicin 2.5 mg/kg every 24 h). A 2.5 French umbilical arterial ca-theter (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 (0.3 ml/h) with 1 IU/ml heparin sodium.

Intensive care support for the animal throughout the 48 h experi-ment and complications (e.g. hypotension, seizures, hyperkalemia) managed in accordance with local neonatal intensive care guidelines. All piglets received continuous physiological monitoring (SA instru-ments). To maintain the MABP above 40 mmHg, bolus infusions of 0.9% saline (Baxter; 10 ml/kg), dopamine (5–20 μg/kg/min), dobuta-mine (5–20 μg/kg/min), noradrenaline (0.1–1 μg/kg/min) and adre-naline (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
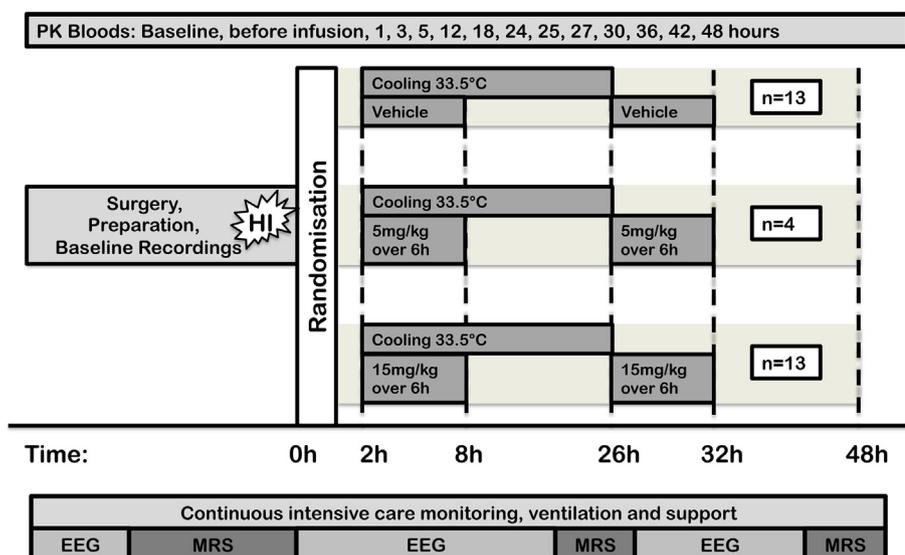
Fig. 1. Study time line. Following baseline data acquisition and screening for health, piglets underwent cerebral hypoxia-ischemia. Following hypoxia-ischemia and cerebral NTP recovery > 70%, piglets were randomized to (i) Hypothermia (33.5 °C from 2 to 26 h) + Vehicle (HT + V); (ii) HT + 5 mg/kg melatonin (Mel-5) at 2 h and 26 h and infused over 6 h; (iii) HT + 15 mg/kg melatonin (Mel-15) at 2 h and 26 h and infused over 6 h. Piglets were maintained under intensive care for 48 h following HI. MRS was acquired at baseline, during HI and 60 min after resuscitation, at 24 and 48 h. aEEG was acquired at baseline and between MRS studies.

temperature was maintained at 33.5 °C from 2 to 26 h using a cooling water mattress (Tecotherm); rewarming occurred at 0.5 °C/h. For the remainder of the study rectal temperature was maintained in the normothermic range (38.0–39.0 °C) using a radiant warmer during surgery and subsequently a heating water mattress (Tecotherm).

2.3. 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 24 h (HT + V n = 11, HT + Mel-5 n = 4, HT + Mel-15 n = 13) and 48 h (HT + V n = 8, HT + Mel-5 n = 4, HT + Mel-15 n = 12) after cerebral HI.

For ^{31}P MRS a 7 cm × 5 cm elliptical transmit-receive MRS surface coil tuned to the ^{31}P resonant frequency was positioned above the head. ^{31}P MRS was acquired with 1 min 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 predominantly ATP and the latter contributes approximately 70% of the NTP signal (Mandel and Edel-Harth, 1966). Thus NTP changes during this experiment predominantly 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, 8 × 8 × 15 mm) and in the deep grey matter centered on both lateral thalami (deep grey 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.

2.4. 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 min. 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 1 h 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) (Fig. 1).

2.5. 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 0.33 ml/h over 6 h for a typical 2 kg piglet; for the HT + Mel-15 group the infusion rate was 1 ml/h over 6 h for a typical 2 kg piglet. The vehicle was administered at 1 ml/ h over 6 h for a typical 2 kg piglet.

2.6. 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 6 h time epochs and mean differences analyzed for significance between study groups. Seizure activity was quantified. Electrographic seizures were treated with Phenobarbitone, initially 20 mg/kg, followed by 10 mg/kg for subsequent seizures, up to a maximum 40 mg/kg.

2.7. Blood and CSF for melatonin pharmacokinetics

Blood was sampled at baseline and 1, 3, 6, 12, 18, 26, 27, 29, 32, 38,

44, 50 h after the first melatonin infusion at 2 h. 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.

2.8. 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 3 pg/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%.

2.9. 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 4 °C in 2% paraformaldehyde for 7 days. Coronal slices (5 mm thick) of the right hemisphere, starting from anterior to the optic chiasma, were embedded in paraffin, sectioned to 8 μm 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 (Fig. 2).

2.9.1. 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 (Fig. 2) by an investigator blind to the treatment group and the average converted into counts per mm^2.
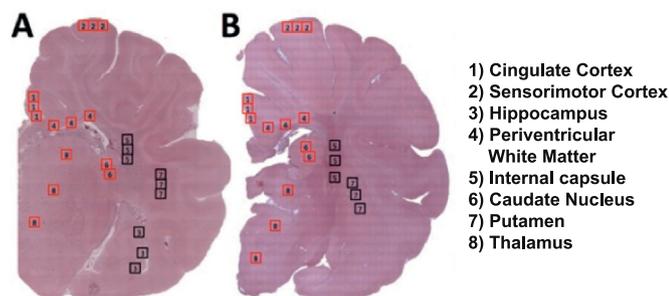
Fig. 2. The two brain levels (A and B) sampled are shown as well as the 8 brain regions assessed for immunohistochemistry (3 fields per region, 2 levels). The red squares represent those areas sampled by MRS and the black squares those regions not sampled by MRS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1) Cingulate Cortex
2) Sensorimotor Cortex
3) Hippocampus
4) Periventricular White Matter
5) Internal capsule
6) Caudate Nucleus
7) Putamen
8) Thalamus

2.9.2. 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.

2.9.3. Iba-1 (sensorimotor and cingulate cortex only)

The activation state of Iba-1 positive cells (Wako 019–19,741, 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.

2.9.4. Cleaved caspase 3 (sensorimotor and cingulate cortex only)

The sections were processed for antigen retrieval (800-mW microwave irradiation in 0.1 M citrate buffer, 10 min), followed by overnight incubation with primary rabbit antibody against activated caspase 3 (Cell signalling 9661 L, dilution 1:100) and then 2 h 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).

2.10. Statistical methods

2.10.1. 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.

2.10.2. Amplitude intergrateded EEG (aEEG)

Following the baseline scoring, scores were obtained hourly until 48 h after HI. Each subject's scores were averaged over 0–6 h, 7–12 h, 13–18 h, 19-24 h, 25–30 h, 31–36 h, 37–42 h 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.

2.10.3. 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.

2.10.4. 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

Table 1

Baseline group data and physiological variables throughout the study.

Parameters	Vehicle Mean (SD)		5 mg/kg/24 h Mean (SD)		15 mg/kg/24 h 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

Time zero was set at the time of reperfusion/resuscitation. Mean ± 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 12 h 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.

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

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 48 h after HI.

Infusions	HT + V		HT + Mel-5		HT + Mel-15		P value
	Mean (SD)		Mean (SD)		Mean (SD)		
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
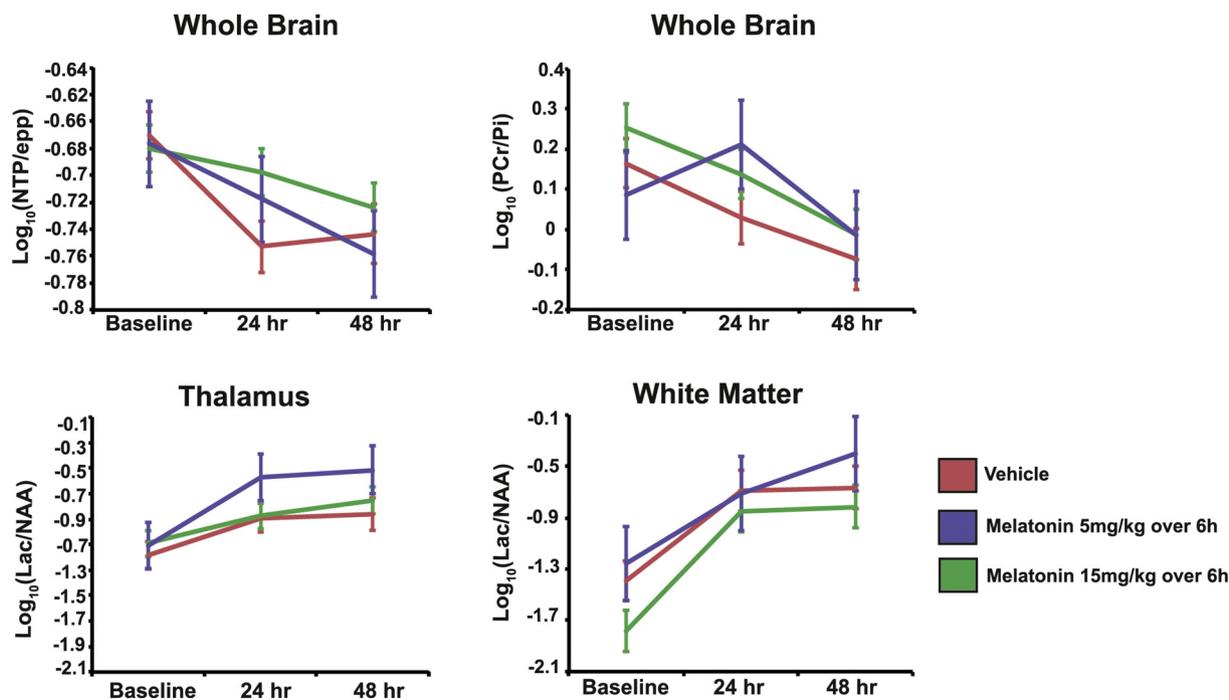
Fig. 3. Magnetic resonance spectroscopy of the brain at baseline, 24 h and 48 h after hypoxia ischemia. All groups underwent therapeutic hypothermia (HT) from 2 to 26 h. Least square mean plots with standard error of the mean (SEM) error bars are shown for (Fig. 3A) NTP/epp and (Fig. 3B) PCr/Pi in whole forebrain and thalamic (Fig. 3C) and white matter (Fig. 3D) Lac/NAA for vehicle, Mel-5 and Mel-15 groups. NTP/epp was higher in the HT + Mel-15 group at 24 h (p = 0.038) but not at 48 h. There were no other differences between groups. Epp = exchangeable phosphate pool; Lac = lactate; NAA = *N*-acetylaspartate; WM = white matter; HI = hypoxia–ischemia. *p < 0.05.

HT + Mel-5 group and 13 animals in the HT + Mel-15 group. The interim analysis (after 20 piglets) suggested futility in continuing the 5 mg/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.

3.1. 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 12 h 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 48 h 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).

3.2. 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 Fig. 3. Comparing HT + V with HT + Mel-5 and HT + Mel-15 groups, NTP/epp was higher in the HT + Mel-15 group at 24 h

(p = 0.038) but not at 48 h (Fig. 3A). There was no difference in PCr/Pi at any time point (Fig. 3B). There was no difference between groups for Lac/NAA in the thalamus (Fig. 3C) and white matter (Fig. 3D) at 24 and 48 h.

3.3. aEEG

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

3.4. TUNEL

The estimated mean TUNEL cells/mm^2 for the treatment groups are shown in Table 3 and Fig. 5. TUNEL positive cells were counted at 2 brain levels (Fig. 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
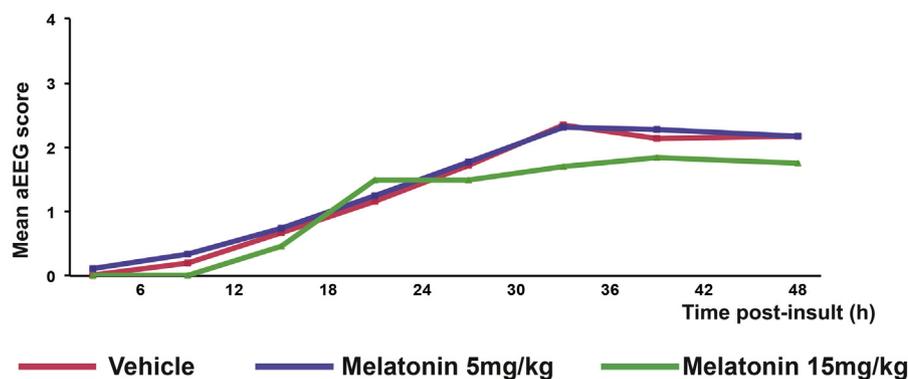
Fig. 4. Amplitude-integrated electroencephalogram (aEEG). Scores were obtained hourly until 48 h and averaged over 6 hourly periods. An ANOVA model was fitted and the differences in the means between the treatment groups estimated with 95% CIs for the differences There was no difference in the group mean hourly aEEG scores between groups. Representative aEEG traces for each score are shown in the lower panel.
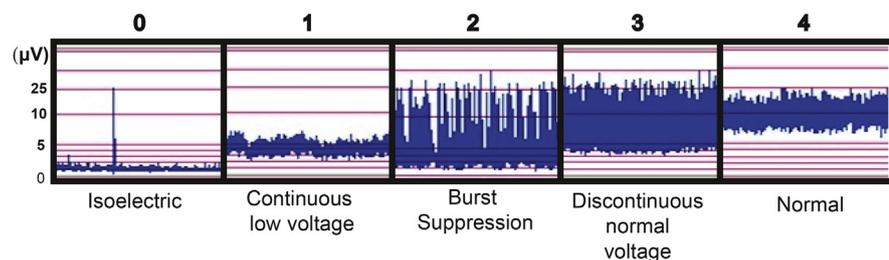
Table 3
Differences between HT + V, HT + Mel-5 and HT + Mel-15 group TUNEL counts for the sensorimotor cortex and overall (8 brain regions). The other brain regions did not show a significant difference in TUNEL counts.

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

Bold denotes a significant difference.

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. 1).

3.5. 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 (Fig. 6).

3.6. Pharmacokinetics

Basal mean (± SD) plasma endogenous melatonin levels were detectable in the HT + V group (C$_{max}$ 0.0014 ± 0.0012 mg/l and AUC$_{last}$

0.015 ± 0.012 mg∗h/l). Mean (± SD) plasma melatonin levels in the HT + Mel-5 group were ~10^3-fold higher (C$_{max}$ 3.97 ± 1.53 mg/l and AUC$_{last}$ 136 ± 61 mg∗h/l). Mean (± 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 18 mg/kg melatonin started at 1 h after HI and given more rapidly over 2 h, would result in putative therapeutic plasma concentrations by 3 h after HI (compared to 8 h after HI in our current study) (Fig. 7).

CSF melatonin levels at 48 h in the HT + V group were low (ca.0.0001 mg/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 48 h) of 0.5 (range 0.3–1.0) in the HT + Mel-15 group demonstrated adequate CNS penetration.

4. 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 15 mg/kg proprietary intravenous melatonin formulation (PCT/EP2018/056423)
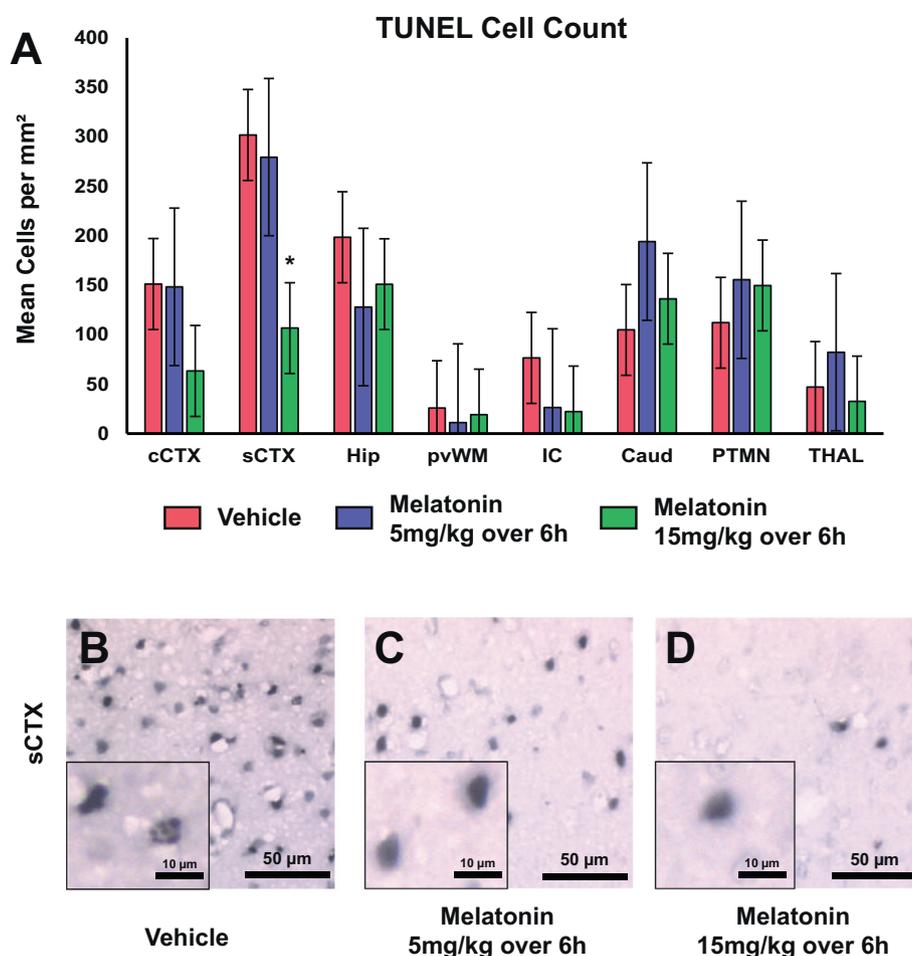
Fig. 5. Mean TUNEL counts for HT + V, HT + Mel-5 and HT + Mel-15. Fig. 5A shows TUNEL counts across 8 different brain regions. There is a significant reduction in TUNEL positive cells in the HT + Mel-15 group compared to HT + V in the sensorimotor cortex (p = 0.003). Representative TUNEL sections of 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. *p < 0.01.

given at 2 h and 26 h 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 (8 h 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 8 h or at any time after HI. The lower dose of melatonin (5 mg/kg at 2 and 26 h), 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 2 h and 26 h combined with cooling from 2 to 26 h. 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 min 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
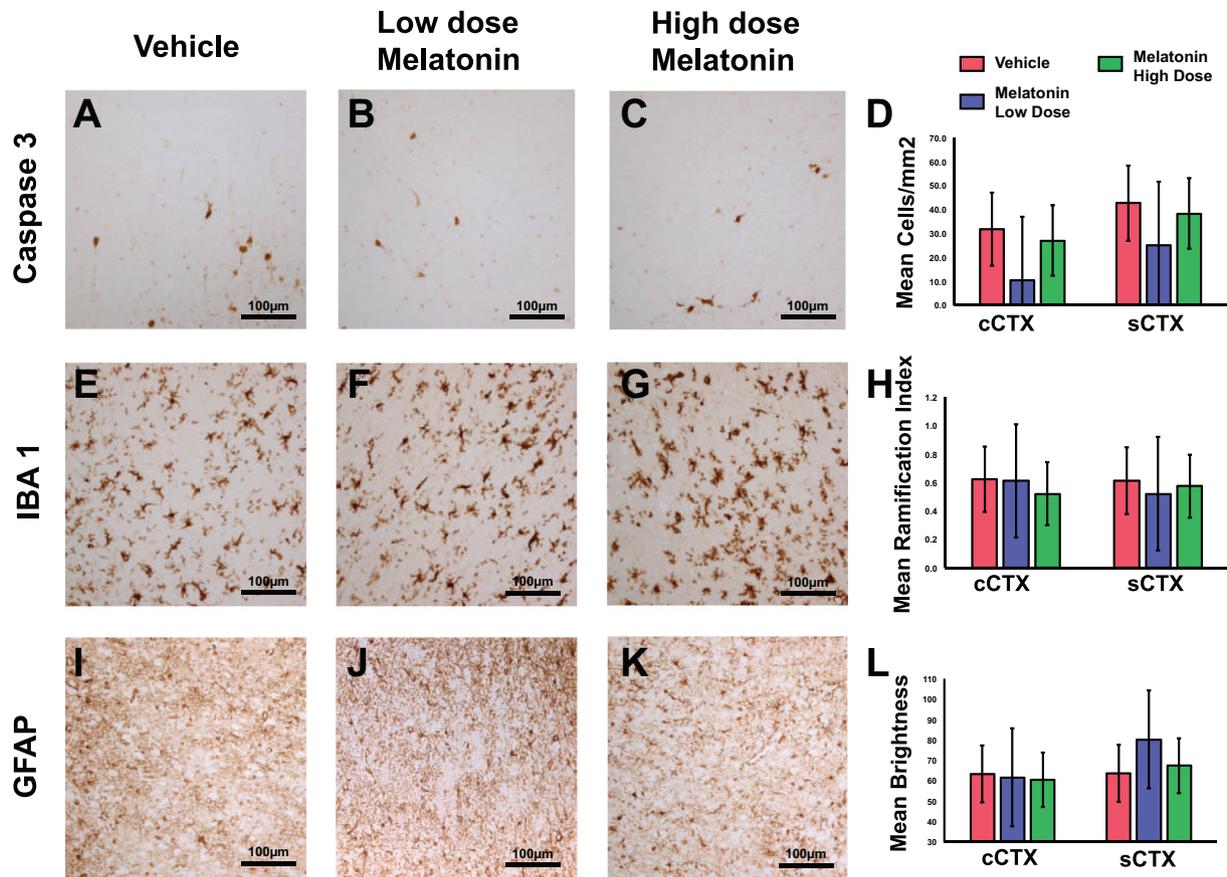
Fig. 6. Immunohistochemistry in the sensorimotor and cingulate cortex in HT + V, HT + Mel-5 and HT + Mel-15 groups. The top panel shows cleaved caspase 3 (CC3), middle panel IBA-1 staining and lower panel GFAP. There was no difference in any of the immunohistochemical markers between groups.

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 48 h; 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 24 h but not 48 h, this may relate to fewer subjects being studied at 48 h. 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 48 h (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 to 26 h with melatonin (5 mg/kg or 15 mg/kg intravenous infusions over 6 h) started 2 h after HI and repeated at 26 h 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 6 h of birth with enteral melatonin at a total dose of 27 mg/kg over 14 h 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 50 mg/kg dose over 5 days, commenced within 6 h. 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 10 mg/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
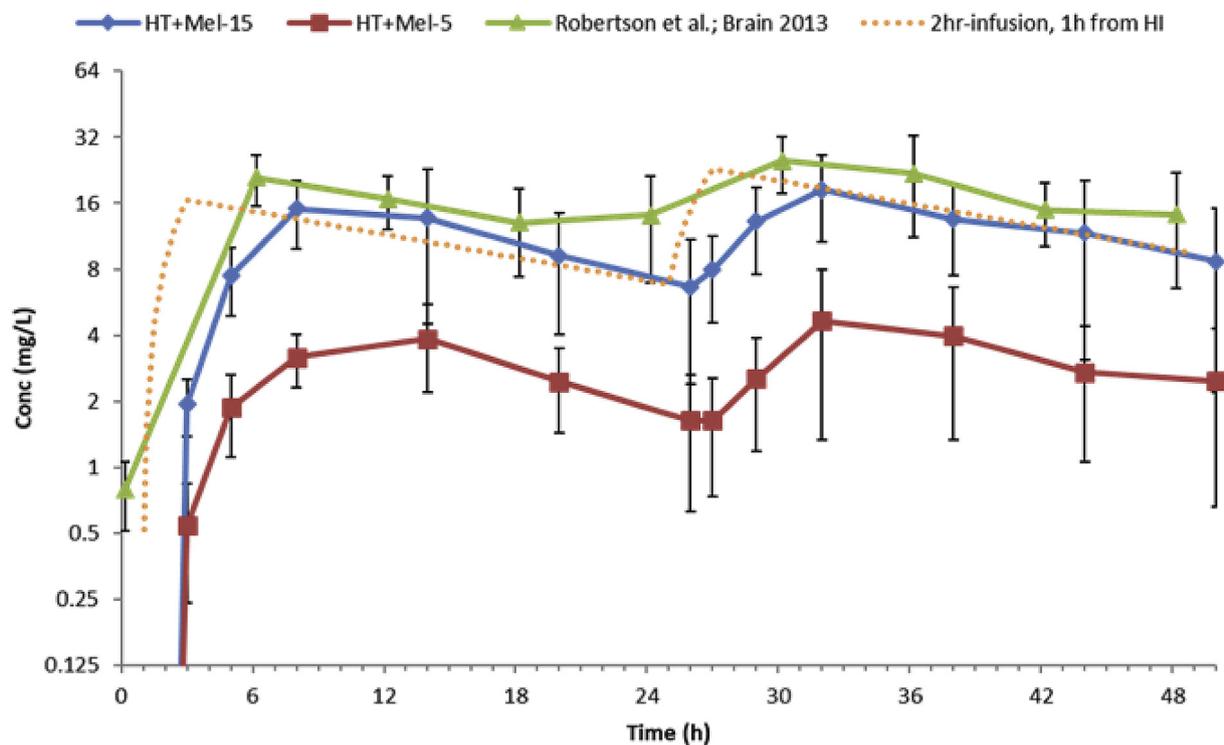
Fig. 7. Mean plasma concentrations (± SD) of the HT + Mel-5 (red line) and HT + Mel-15 groups (blue line) compared with the historical data from Robertson et al., 2013 (green line); plasma concentrations of the HT + V group (< 0.125 mg/l) could not be plotted due to the Y axis scale used. PK modelling for a proposed dose of 18 mg/kg infused over 2 h and started at 1 h 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 $t = 0$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 8 h after HI – an increase of $\sim 10^4$. 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 15–30 mg/l and earlier attainment of therapeutic levels within 2–3 h after cerebral HI are necessary for maximal neuroprotection. In a fetal sheep model of preterm HI, a continuous melatonin infusion from 2 to 26 h (0.2 mg intravenous melatonin bolus followed by 0.1 mg/h for the next 24 h with ethanol excipient) led to a 70-fold increase in plasma levels at 10 h compared to baseline (pre-melatonin infusion values; 10.1 ± 4.4 pg/ml, peak levels 1717 ± 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 60 mg melatonin started at 30 min 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 60 mg dose given over 24 h. 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 again unclear (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 previous perinatal asphyxia piglet study (Robertson et al., 2013). A study in fetal sheep with low-dose melatonin (0.1 mg/kg over 6 h) 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 4 h 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 8 h 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 ~10 h after birth had no benefit (Azzopardi et al., 2016). Pharmacokinetic modelling suggests that 18 mg/kg melatonin started at 1 h after HI and given over 2 h would achieve levels within the therapeutic range by 3 h; this protocol needs to be assessed in future safety and efficacy studies. The 15 mg/kg dose was the primary target in this study, based on previous studies (Robertson et al., 2013); fewer piglets were studied in the 5 mg/kg group than the 15 mg/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 12 h 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 HT 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 2 h 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 min 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 48 h 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 15 mg/kg proprietary intravenous melatonin formulation given 2 h and 26 h after cerebral HI in a piglet model of NE. Plasma melatonin levels increased ~10^4 by 8 h after HI with 15 mg/kg intravenous proprietary melatonin formulation, reaching the putative target therapeutic range at 8 h and remaining at this level for 48 h. A lower dose of melatonin (5 mg/kg at 2 and 26 h) 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 18 mg/kg melatonin given intravenously at 1 h after HI with a faster infusion rate (over 2 h) is likely to reach putative therapeutic concentrations by 2–3 h (compared to 8 h 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2018.10.004.

Funding

References

randomized controlled pilot study. J. Perinatol. 35, 186–191.

Aridas, J., et al., 2018. Systemic and transdermal melatonin administration prevents neuropathology in response to perinatal asphyxia in newborn lambs. J. Pineal Res. https://doi.org/10.1111/jpi.12479. (Epub ahead of print).

Aronowski, J., et al., 2003. Ethanol plus caffeine (caffeinol) for treatment of ischemic stroke: preclinical experience. Stroke 34, 1246–1251.

Azzopardi, D., et al., 1989. Prognosis of newborn infants with hypoxic-ischemic brain injury assessed by phosphorus magnetic resonance spectroscopy. Pediatr. Res. 25, 445–451.

Azzopardi, D., et al., 2016. Moderate hypothermia within 6h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-Xe): a proof-of-concept, open-label, randomized controlled trial. Lancet Neurol. 15, 145–153.

Boschen, K., Klintsova, A., 2017. Neurotrophins in the brain:interaction with alcohol exposure during development. Vitam. Horm. 104, 197–242.

Brzezinski, A., 1997. Melatonin in humans. N. Engl. J. Med. 336, 186–195.

Buscemi, N., et al., 2006. Efficacy and safety of exogenous melatonin for secondary sleep disorders and sleep disorders accompanying sleep restriction: meta-analysis. BMJ 332, 385–393.

Carloni, S., et al., 2018. Melatonin acts in synergy with hypothermia to reduce oxygen-glucose deprivation (OGD) in rat hippocampus organotypic slice cultures. Neonatology 114 (4), 364–371.

Cho, B., Toledo-Pereyra, L., 2008. Caspase-independent programmed cell death following ischemic stroke. J. Investig. Surg. 21, 14107.

Csekő, A., et al., 2013. Accuracy of amplitude-integrated electroencephalography in the prediction of neurodevelopmental outcome in asphyxiated infants receiving hypothermia treatment. Acta Paediatr. 102, 707–711.

Drury, P., et al., 2014. Partial neural protection with prophylactic low-dose melatonin after asphyxia in preterm fetal sheep. J. Cereb. Blood Flow Metab. 34, 126–135.

Edwards, D., et al., 2010. Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. BMJ 340, C363. https://doi.org/10.1136/bmj.c363.

Faulkner, S., et al., 2011. Xenon augmented hypothermia reduces early lactate/NAA and cell death in Perinatal Asphyxia. Ann. Neurol. 70, 133–150.

Fraser, S., et al., 1983. Direct radioimmunoassay and gas chromatography-mass spectrometry compared for determination of melatonin in plasma. Clin. Chem. 29, 1703–1704.

Fulia, F., et al., 2005. Increased levels of malondialdehyde and nitrite/nitrate in the blood of asphyxiated newborns: reduction by melatonin. J. Pineal Res. 31, 343–349.

Galano, A., et al., 2011. Melatonin as a natural ally against oxidative stress: a physico-chemical examination. J. Pineal Res. 51, 1–16.

Galano, A., et al., 2013. On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. J. Pineal Res. 54, 245–257.

Gitto, E., et al., 2001. Effects of melatonin treatment in septic newborns. Pediatr. Res. 50, 756–760.

Gitto, E., et al., 2004a. Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: beneficial effects of melatonin. Am. J. Perinatol. 21, 209–216.

Gitto, E., et al., 2004b. Melatonin reduces oxidative stress in surgical neonates. J. Pediatr. Surg. 39, 184–189.

Gitto, E., et al., 2013. Protective role of melatonin in neonatal diseases. Oxidative Med. Cell. Longev. 2013, 980374.

Hellström-Westas, L., et al., 1995. Predictive value of early continuous amplitude integrated EEG recordings on outcome after severe birth asphyxia in full term infants. Arch. Dis. Child. Fetal Neonatal Ed. 72, F34–F38.

Husson, I., et al., 2002. Melatoninergic neuroprotection of the murine periventricular white matter against neonatal excitotoxic challenge. Ann. Neurol. 51, 82–92.

Jacobs, S., et al., 2011. Whole-body hypothermia for term and near-term newborns with hypoxic-ischemic encephalopathy: a randomized controlled trial. Arch. Pediatr. Adolesc. Med. 165, 692–700.

Jahnke, G., et al., 1999. Maternal and developmental toxicity evaluation of melatonin administered orally to pregnant Sprague-Dawley rats. Toxicol. Sci. 50, 271–279.

Kilic, U., et al., 2005. Signal transduction pathways involved in melatonin-induced neuroprotection after focal cerebral ischemia in mice. J. Pineal Res. 38, 67–71.

Klintsova, A., et al., 2007. Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. Alcohol. Clin. Exp. Res. 31, 2073–2082.

Kurinczuk, J., et al., 2010. Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. Early Hum. Dev. 86, 329–338.

Lee, A., et al., 2013. Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. Pediatr. Res. 74, 50–72.

Leon, J., et al., 2004. Melatonin and mitochondrial function. Life Sci. 75, 765–790.

Liu, L., et al., 2016. Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the Sustainable Development Goals. Lancet 388, 3027–3035.

Lorek, A., et al., 1994. Delayed ("secondary") cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. Pediatr. Res. 36, 699–706.

Lowes, D., et al., 2013. Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. Br. J. Anaesth. 110, 472–480.

Luchetti, F., et al., 2010. Melatonin signaling and cell protection function. FASAB J. 24, 3603–3624.

Macleod, M., et al., 2005. Systematic review and meta-analysis of the efficacy of melatonin in experimental stroke. J. Pineal Res. 38 (1), 35–41 2005 Jan.

Mandel, P., Edel-Harth, S., 1966. Free nucleotides in the rat brain during post-natal

development. J. Neurochem. 13, 591–595.

Marek, E., Kraft, W., 2014. Ethanol pharmacokinetics in neonates and infants. Curr. Ther. Res. Clin. Exp. 76, 90–97.

Miller, S., et al., 2005. Melatonin provides neuroprotection in the late-gestation fetal sheep brain in response to umbilical cord occlusion. Dev. Neurosci. 27, 200–210.

Mitra, S., et al., 2018. Proton magnetic resonance spectroscopy lactate/N-acetylaspartate within 2 weeks of birth accurately predicts 2-year motor, cognitive and language outcomes in neonatal encephalopathy after therapeutic hypothermia. Arch. Dis. Child. Fetal. Neonatal. Ed. https://doi.org/10.1136/archdischild-2018-315478.

NPEU, MBRRACE-UK, 2017. Mothers and Babies: Reducing Risk from Audits and Confidential Enquiries Across the UK. https://www.npeu.ox.ac.uk/mbrrace-uk.

Paradies, G., et al., 2010. Melatonin, cardiolipin and mitochondrial bioenergetics in health and disease. J. Pineal Res. 48, 297–310.

Penrice, J., et al., 1997. Proton magnetic resonance spectroscopy of the brain during acute hypoxia-ischemia and delayed cerebral energy failure in the newborn piglet. Pediatr. Res. 41, 795–802.

Reiter, R., et al., 2016. Melatonin as an antioxidant: under promises but over delivers. J. Pineal Res. 61, 253–278.

Robertson, N., et al., 2012. Which neuroprotective agents are ready for bench to bedside translation in the newborn infant? J. Pediatr. 160, 544–552.

Robertson, N., et al., 2013. Melatonin augments hypothermic neuroprotection in a perinatal asphyxia piglet model. Brain 136 (Pt 1), 90–105.

Seifman, M., et al., 2008. Endogenous melatonin increases in cerebrospinal fluid of patients after severe traumatic brain injury and correlates with oxidative stress and metabolic disarray. J. Cereb. Blood Flow Metab. 28, 684–696.

Shankaran, S., et al., 2014. Effect of depth and duration of cooling on deaths in the NICU among neonates with hypoxic ischemic encephalopathy: a randomized clinical trial.

JAMA 312, 2629–2639.

Shankaran, S., et al., 2017. Effect of depth and duration of cooling on death or disability at age 18 months among neonates with hypoxic-ischemic encephalopathy: a randomized clinical trial. JAMA 318, 57–67.

Skaper, S., et al., 1998. Melatonin prevents the delayed death of hippocampal neurons induced by enhanced excitatory neurotransmission and the nitridergic pathway. FASEB J. 12, 725–731.

Su, F., et al., 2017. Low-dose ethanol preconditioning protects against oxygen-glucose deprivation/reoxygenation-induced neuronal injury by activating large conductance, Ca2+-activated K+ channels in vitro. Neurosci. Bull. 33, 28–40.

Thoresen, M., et al., 2013. Time is brain: starting therapeutic hypothermia within three hours after birth improves motor outcome in asphyxiated newborns. Neonatology 104, 228–233.

van Rooij, L., et al., 2005. Recovery of amplitude integrated electroencephalographic background patterns within 24 hours of perinatal asphyxia. Arch. Dis. Child. Fetal Neonatal Ed. 90, F243–F251.

Vanhamme, L., et al., 1997. Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. J. Magn. Reson. Imaging 129, 35–43.

Wang, F., et al., 2012. Neuroprotective effect of acute ethanol administration in a rat with transient cerebral ischemia. Stroke 43, 205–210.

Welin, A., et al., 2007. Melatonin reduces inflammation and cell death in white matter in the mid-gestation fetal sheep following umbilical cord occlusion. Pediatr. Res. 61, 153–158.

Whittaker, A., et al., 2009. Toxic additives in medication for preterm infants. Arch. Dis. Child. Fetal Neonatal Ed. 94, F236–F240.

Yawno, T., et al., 2017. The beneficial effects of melatonin administration following hypoxia-ischemia in preterm fetal sheep. Front. Cell. Neurosci. 11, 296.