Melatonin reduces brain injury following inflammation-amplified hypoxia-ischemia in a translational newborn piglet study of neonatal encephalopathy

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Melatonin reduces brain injury following inflammation-amplified hypoxia–ischemia in a translational newborn piglet study of neonatal encephalopathy

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Abstract

There is a need to develop therapies for neonatal encephalopathy (NE) in low- and middle-income countries (LMICs) where the burden of disease is greatest and therapeutic hypothermia (HT) is not effective. We aimed to assess the efficacy of melatonin following inflammation-amplified hypoxia–ischaemia (IA-HI) in the newborn piglet. The IA-HI model accounts for the contribution of infection/inflammation in this setting and HT is not cytoprotective. We hypothesised that intravenous melatonin (5% ethanol, at 20 mg/kg over 2 h at 1 h after HI + 10 mg/kg/12 h between 24 and 60 h) is safe and associated with: (i) reduction in magnetic resonance spectroscopy lactate/*N*-acetylaspartate (MRS Lac/sNAA); (ii) preservation of phosphorus MRS phosphocreatine/phosphate exchange pool (PCr/Epp); (iii) improved aEEG/EEG recovery and (iv) cytoprotection on immunohistochemistry. Male and female piglets underwent IA-HI by carotid artery occlusion and reduction in FiO_2 to 6% at 4 h into *Escherichia coli* lipopolysaccharide sensitisation (2 μg/kg bolus + 1 μg/kg/h over 12 h). At 1 h after IA-HI, piglets were randomised to HI-saline ($n = 12$) or melatonin ($n = 11$). There were no differences in insult severity between groups. Target melatonin levels (15–30 mg/L) were achieved within 3 h and blood ethanol levels were <0.25 g/L. At 60 h, compared to HI-saline, melatonin was associated with a reduction of 0.197 $\log_{10}$ units (95% CrI [−0.366, −0.028], $Pr_{(sup)}$ 98.8%) in basal-ganglia and thalamic Lac/NAA, and 0.257 (95% CrI [−0.676, 0.164], $Pr_{(sup)}$ 89.3%) in white matter Lac/NAA. PCr/Epp was higher in melatonin versus HI-saline ($Pr_{(sup)}$ 97.6%). Melatonin was associated with earlier aEEG/EEG recovery from 19 to 24 h ($Pr_{(sup)}$ 95.4%). Compared to HI-saline, melatonin was associated with increased NeuN+ cell

density ($Pr_{(sup)}$ 99.3%) across five of eight regions and reduction in TUNEL-positive cell death ($Pr_{(sup)}$ 89.7%). This study supports the translation of melatonin to early-phase clinical trials. Melatonin is protective following IA-HI where HT is not effective. These data guide the design of future dose-escalation studies in the next phase of the translational pipeline.

KEYWORDS

hypoxia–ischaemia, inflammation, melatonin, neonatal encephalopathy, neuroprotection

1 | INTRODUCTION

Neonatal encephalopathy (NE) is a leading cause of mortality and morbidity across the world, affecting 1.4 million babies every year.[1] The burden of disease is greatest in low-resource settings, with 85% of cases in sub-Saharan Africa and Southeast Asia.[1] Therapeutic hypothermia (HT) is the only effective cerebroprotective therapy currently available, with an associated number needed to treat is 7–8[2] in the high-resource setting. Recently, in a large multicentre randomised control trial (RCT) of babies across India, Bangladesh and Sri Lanka, HT was associated with an increase in mortality and no reduction in disability.[3] These findings realign the focus and urgent need to identify alternative therapies effective for babies with NE in low- and middle-income countries (LMICs).

Several factors may contribute to the lack of benefit of HT in LMICs, including perinatal infection and inflammation. Tann et al.[4] reported that neonatal bacteraemia and histological funisitis independently increase the risk of NE in Uganda. In our established piglet model of NE,[5] prior inflammation sensitisation with *Escherichia coli* lipopolysaccharide (LPS) followed by hypoxia–ischaemia (HI) was associated with increased mortality and TUNEL-positive cell death, particularly in the white matter and cortical regions.[5] Importantly, HT for 24 h did not improve neurological outcomes based on magnetic resonance spectroscopy (MRS) of lactate to *N*-acetyl aspartate peak area ratio (Lac/NAA), background amplitude-integrated encephalography (aEEG/EEG) activity and immunohistochemistry.[6] Similar findings have been reported by other groups[7–9] with evidence of a pathogen-specific response.[10]

Melatonin (*N*-acetyl-5-methoxytryptamine) for NE has shown great promise in preclinical studies. Cytoprotective actions include free radical scavenging activity, antiapoptotic and anti-inflammatory properties. In a recent meta-analysis of preclinical studies of NE, melatonin was associated with significant reduction in brain infarct size, improved neurobehavioural outcomes and cell death reduction.[11] The improvement in neurological outcomes occurred both with melatonin monotherapy and as an adjunct with HT. Preclinical evidence thus demonstrates robust protection following HI; however, the benefit with IA-HI is unknown.

The lipophilic nature of melatonin allows easy penetration across the blood–brain barrier, but limits its solubility in water. To date, no intravenous formulation of melatonin is available for clinical use. Excipients used in preclinical studies include ethanol,[12–17] cyclodextrin-derivates,[18,19] dimethylsulphoxide (DMSO)[20–23] and tween[24] to enhance solubility; however, their individual safety profiles need careful consideration. Improved outcomes in ethanol-containing melatonin formulations are reported[11]; partial cytoprotection was seen in piglets[12] and preterm lambs[25] receiving low-dose ethanol. The United Kingdom Medicines Health Regulatory Authority do not stipulate absolute blood alcohol concentration (BAC) limits in medicinal products; however, the American Academy of Paediatrics Committee on Drugs recommend levels should not exceed 0.25 g/L after a single dose.[26] In this study, we have optimised melatonin administration to limit ethanol exposure while achieving target melatonin levels.

This translational study aimed to assess safety and efficacy of an optimised intravenous melatonin regimen as a monotherapy under normothermia in a piglet model of NE relevant to the low-resource setting. We hypothesised that intravenous melatonin (5% ethanol, at 20 mg/kg over 2 h at 1 h after HI + 10 mg/kg/12 h between 24 and 60 h) is cytoprotective and associated with: (i) reduction in MRS Lac/NAA; (ii) preservation of phosphorus (^{31}P) MRS phosphocreatine/exchangeable phosphate pool (PCr/Epp); (iii) improved aEEG/EEG recovery and (iv) cytoprotection on immunohistochemistry. The immunohistochemistry markers assessed included terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), neuronal nuclear protein (NeuN), ionised calcium-binding adapter molecule-1 (Iba-1), oligodendrocyte transcription factor-2 (Olig2), and glial fibrillary acidic protein (GFAP).

2 | MATERIALS AND METHODS

The study was approved by the University College London ethics committee, conducted in accordance with the UK Home Office Regulations [Animals (Scientific Procedures) Act 1986] and reported under the ARRIVE 2.0 guidance.

2.1 | Sample size

In our previous neuroprotection piglet studies, we observed treatment differences between 0.5 and 0.77[19,27,28] $\log_{10}$ units in the Lac/NAA peak ratio with a standard deviation of 0.3–0.4. A similar variance was observed in our IA-HI model (SD 0.34–0.42).[5,6] Based on a pessimistic prediction of 0.5 Log_{10} unit reduction in basal ganglia and thalamic (BGT) Lac/NAA and a standard deviation of 0.4, 12 piglets per group were required to achieve 80% power at a 5% type-I error rate.

2.2 | Study design, eligibility criteria and randomisation

The experimental protocol is shown in Figure 1. Newborn male and female Large White piglets were assessed using the following eligibility criteria: (i) good baseline health, (ii) normal baseline aEEG/EEG following surgery, and (iii) isoelectric aEEG/EEG within the first hour of IA-HI. Piglets were randomised using a blinded computer-generated random allocation process at 1 h to receive intravenous (i) 0.9% sodium chloride (HI-saline) or (ii) melatonin.

2.3 | Animal care, surgical preparation and neurocritical care management

Piglets underwent surgical preparation as previously described and transferred to a purpose-built MR-compatible incubator for neonatal neurocritical care.[19] Normothermia was maintained at a rectal temperature of

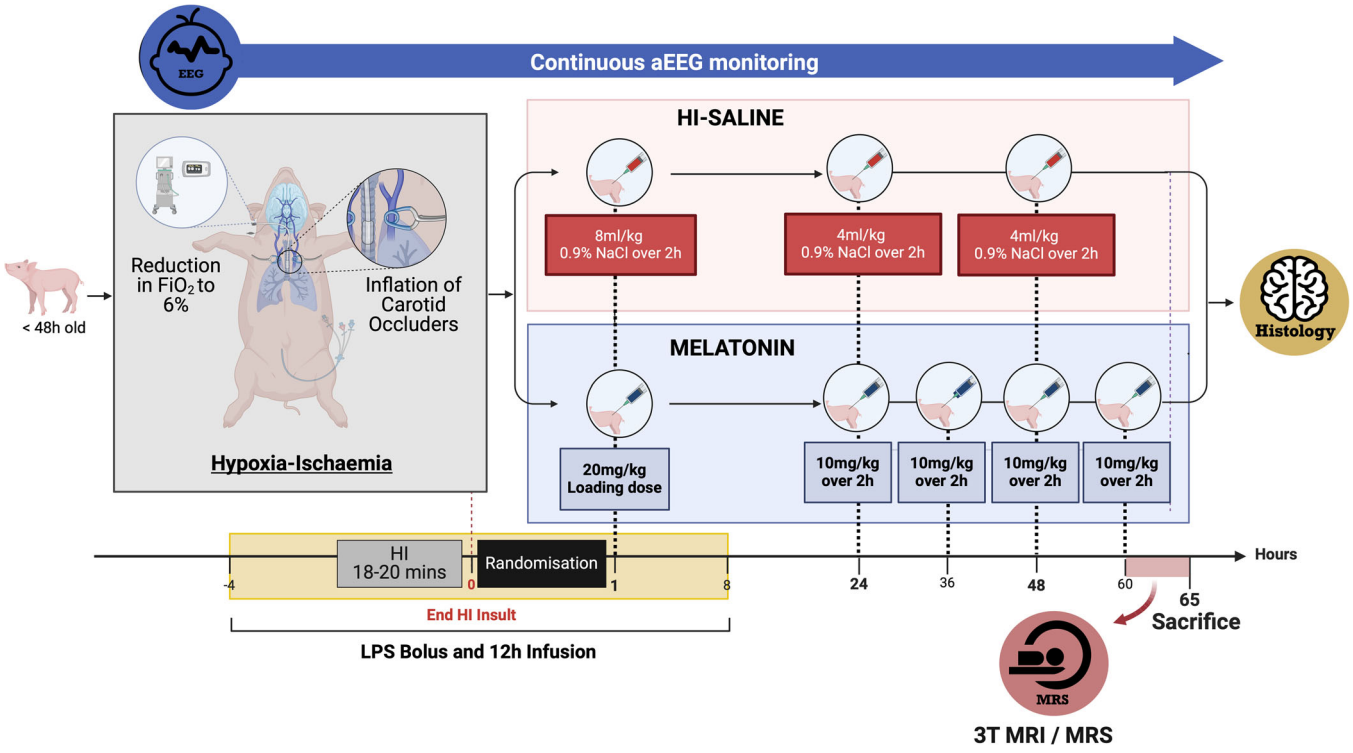

FIGURE 1 Experimental Protocol. In brief, newborn (male and female) large white piglets were examined for good health and surgically prepared as previously described.[6,19] Inflammation-amplified hypoxia–ischaemia (IA-HI) was achieved by *Escherichia coli* liposaccharide (LPS) sensitisation (bolus of 2μg/kg bolus + 1μg/kg/h for 12 h) and hypoxia–ischaemia 4 h into LPS infusion (reduction in FiO2% to 6% and inflation of carotid artery occluders). At 1 h following IA-HI, piglets meeting the eligibility to enter the study were randomised to receive (i) 0.9% sodium chloride (HI-saline) ($n = 12$) or (ii) melatonin (loading dose 20 mg/kg, maintenance dose 10 mg/kg every 12 h after 24 h) ($n = 11$). All piglets received full neonatal neurocritical care management for the duration of the experiments, including continuous aEEG/EEG monitoring and 3 T MRI/MRS at 62–65 h. After 65 h, piglets were euthanised and brain dissected for immunohistochemistry.

38°C using a servo-controlled system (Criticool, Belmont Medica). Complications of neonatal HI were treated following neonatal intensive care guidelines as previously described.[19]

2.4 | Inflammation-amplified hypoxia–ischaemia

Piglets underwent inflammation sensitisation with an intravenous bolus of 2μg/kg *Escherichia coli* LPS O55:B5 (Sigma-Aldrich) followed by a continuous infusion of 1μg/kg/h for 12 h. At 4 h into the LPS infusion, piglets underwent an IA-HI insult.[19] The carotid artery occluders were inflated and FiO_2 was titrated to 6% in a stepwise manner over 3 min. The duration of the HI insult was determined in real time by the blood lactate (target 10–12 mmol/L), duration of isoelectric aEEG and duration of hypotension (mean arterial blood pressure [MABP] 27–30 mmHg). In severe hypotension (MABP < 27 mmHg), the FiO2 was titrated up by 1% every minute until MABP > 27 mmHg was achieved. Following HI, piglets were resuscitated with ventilation breaths in air.

2.5 | Cytoprotective interventions

Melatonin (100 mg) (Sigma-Aldrich) was dissolved in 100% ethanol (2 mL) in the dark and added to 5% glucose to a final concentration of 2.5 mg/mL (5% v/v ethanol) for infusion. A melatonin loading dose of 20 mg/kg (ethanol 320 mg/kg) was administered intravenously over 2 h at 1 h after IA-HI via a central venous line sited in the antecubital fossa. Maintenance doses of 10 mg/kg over 2 h were administered every 12 h from 24 to 60 h. The comparison (HI-saline) group received the equivalent intravenous 0.9% sodium chloride infusion at the same volume and rate.

2.6 | Amplitude-integrated electroencephalography (aEEG/EEG)

Continuous multichannel 6-lead aEEG/EEG (Nicolet, Natus) was acquired over the full duration. At the end of the study, the hourly background aEEG/EEG activity was classified by two lab members blinded to the study group using the voltage criteria described by Hellstrom-Westas.[29] An isoelectric trace was scored 0 and a normal voltage trace scored 4. Seizures were detected by a rise in baseline on aEEG followed by a review of the raw EEG trace demonstrating evolving, repetitive spike and/or wave pattern lasting at least 10 s. Treatment of seizures followed standard neonatal seizure guidelines.[30]

2.7 | Magnetic resonance spectroscopy

^{1}H and ^{31}P MRS were acquired by medical physicists blinded to the treatment group at 60 h using a clinical 3-Tesla MRI scanner (Philips Achieva) as previously described.[19] ^{1}H MRS was acquired using chemical shift imaging with an 8×8 matrix in two $8 \times 8 \times 10\ mm^3$ voxels: the BGT voxel over the left thalamic region and the white matter (WM) voxel at the level of the centrum semiovale. spectral acquisition was acquired with a relaxation time of 2000 ms and a long echo time (TE) of 288 ms as standard. Final post-imaging processing was carried out at the end of the study using TARQUIN and the Lac/NAA peak area ratio was calculated (lactate+threonine/*N*-acetylaspartate+*N*-acetylaspartylglutamate). The ^{31}P MRS metabolites were acquired using a 6-cm diameter circular transmit-receive coil (PulseTeq) placed above the piglet's head with a single-pulse acquisition at an average repetition time of 10 and 32 s.

2.8 | Immunohistochemistry

Piglets were euthanised at 65 h with intravenous pentobarbital followed immediately by intracardiac cold phosphate-buffered saline perfusion and 5% paraformaldehyde (PFA). The brain was subsequently dissected, immersed in 4% PFA for 1 month and then embedded in paraffin. For immunohistochemistry, 8 μm sections were cut from two coronal slices at the level of the optic chiasm and hippocampus from the right hemisphere. Slides were stained for TUNEL+ cell density for cell death, NeuN+ cell density for neuronal survival, Iba-1 cell count for neuro-inflammation, OLIG2+ cell density for oligodendrocyte survival and GFAP for astrocytosis (see Supporting Information for detailed methodology).

2.9 | Biochemical assays

Melatonin, ethanol and acetaldehyde concentrations were measured on serum and brain tissue samples by NovoLytiX GmbH (see Supporting Information). For melatonin and ethanol pharmacokinetic analysis, serum blood samples were taken at 1, 1.5, 2, 3, 7, 13, 24, 25, 26, 30, 36, 48, 49, 50, 54, 60, 62 and 65 h after HI.

Serum cytokines (interleukin-1ra [IL-1ra], IL-4, IL-6, IL-10, IL-12) levels were measured using a porcine-specific, Luminex Discovery Multi-analytes Assay (Bio-Techne, R&D Systems) following the manufacturer's instructions. Full blood count was measured by the Clinical Pathology Laboratory at the Royal Veterinary College. The systemic inflammation response index (SIRI) and systemic immune-inflammation index (SII) were deduced using the formulae, neutrophils × monocytes/lymphocytes and platelets × neutrophils/lymphocytes, respectively.

2.10 | Statistical analysis

Statistical analysis was performed using JMPv14 (SAS), R-software (v4.0.5) and GraphPad Prism (v9). Physiological data, biochemical and haematological results were assessed using an ANOVA model with fixed factor effects of treatment, time and treatment × time interaction plus a random effect subject to account for repeated measures where necessary as previously described.[19] Treatment groups were compared using 95% confidence interval (CI) for the difference in the least square (LS) means and p values deduced. A Bayesian statistical approach was used to assess neurological outcomes (MRS, aEEG and histology) to determine the probability of treatment benefit using non-informative priors. Unlike frequentist statistical methods,[31–33] the Bayesian approach avoids the dichotomisation of results, enables direct hypothesis testing (such as probability of treatment superiority) and therefore produces inferences that are more intuitive and meaningful for decision-making purposes. Detailed methodology is included in the Supporting Information. At the final analysis, a $Pr_{(sup)}$ of 94.8% was considered the threshold for treatment superiority.

We used the *'nlmxr2'* package in R (v4.3.1) to fit a population-based nonlinear mixed-effects model to the melatonin pharmacokinetic data. Age (hours from the insult) and weight were co-variants tested and included in the final model. Graphical inspection of goodness of fit was evaluated and shown in the Supporting Information.

3 | RESULTS

3.1 | Baseline characteristics

The baseline characteristics and insult parameters are shown in Table 1. Twenty-four animals were recruited to enter the study; however, one animal in the melatonin arm was excluded a priori due to severe hyperglycaemia before the IA-HI insult, contributing to brain injury. Twenty-three animals were studied and randomised to either (i) HI-saline ($n = 12$) or (ii) melatonin ($n = 11$). Physiological and biochemical parameters for the IA-HI insult were similar across the groups ($p > .05$) (Table 1).

3.2 | Physiological response

We observed no significant differences in the heart rate, MABP and temperature between the two groups throughout the experiment except a higher temperature at baseline in the melatonin arm (LS mean difference 0.5, 95% CI [0.2, 0.7]} (Supporting Information: Table), which normalised at subsequent time points. The mean vasoactive inotropic score (VIS) to maintain MABP ≥40 mmHg was higher in melatonin-treated animals at 3-6 h (+4.3, 95% CI [0.3, 6.6]) and 6–12 h (+3.8, 95% CI [0.6, 6.9]) (Figure 2).

3.3 | Pharmacokinetics

Serum melatonin, ethanol and acetaldehyde levels are shown in Figure 3. Following an intravenous loading dose of 20 mg/kg melatonin over 2 h, the peak serum melatonin (mean ± SD) level was 33.5 ± 7.6 mg/L at 3 h after IA-HI with a trough of 14.0 ± 8.1 mg/L at 24 h. Peak serum levels were ~10^4-fold higher compared to HI-saline animals (~0.4 μg/L). Subsequent maintenance doses of 10 mg/kg every 12 h achieved peak serum levels of 26.4 ± 12.6 to 27.7 ± 12.0 mg/L. The median melatonin level in the brain tissue ($n = 6$) was ~10^3-fold higher compared to HI-saline.

A population-based pharmacokinetic model was derived using a one-compartment model and the PK parameter estimates are shown in Table 2. The inclusion of age and weight covariates with the combined additive and proportional error provided the best fit as demonstrated by a reduction in the objective function value (OFV). This was used as the final model with the goodness of fit plots shown in the Supporting Information. As shown in Table 2, the parameter estimates for clearance (CL), volume distribution (Vd) and half-life were 0.086 L/h, 1.23 L and 10 h, respectively.

The peak BAC was 0.189 ± 0.041 g/L at 3 h after IA-HI following the loading dose and ranged from 0.041 ± 0.024 to 0.053 ± 0.024 g/L with maintenance doses (see Figure 3C). Acetaldehyde measured in six animals did not accumulate (see Figure 3D). The peak acetaldehyde concentration in the melatonin-treated group was 0.46 ± 0.12 mg/L with the loading dose, and subsequently fell rapidly within 3 h to levels similar to the HI-saline group.

TABLE 1 Baseline and insult characteristics.

	HI-saline ($n = 12$)	Melatonin ($n = 11$)	p Value
Male sex (n, %)	6 (50%)	5 (45%)	
Weight (kg)	2 (2–2.1)	2 (1.9–2)	0.562
Age (h)	51.5 (42.6–60.5)	46.7 (37.7–55.6)	0.430
IA-HI parameters (LS means [95% CI])			
Duration (min)	18.3 (17.3–19.2)	17.1 (16.2–18)	0.080
FiO_2 (AUC)	262 (241.6–282.4)	240.6 (220.2–261)	0.136
Physiological			
Duration isoelectric EEG/aEEG trace (min)	15.2 (14.5–15.9)	14.3 (13.6–15)	0.070
Duration MABP < 30 mmHg (min)	5.1 (2.9–7.3)	5.9 (3.7–8)	0.600
Duration MABP < 27 mmHg (min)	0.6 (−0.5 to 0.5)	1.2 (0.1–2.3)	0.477
NIRS: SaO_2 (AUC)	410.7 (336–485.4)	387.7 (314.5–460.9)	0.647
Biochemical (end of HI insult)			
pH	7.27 (7.21–7.34)	7.27 (7.20–7.33)	0.894
Base excess (mEq/L)	−7.4 (019.2–1.3)	−8.6 (−11.4−−5.8)	0.521
Lactate (mmol/L)	10.6 (9–12.2)	12.2 (10.6–13.7)	0.160
Creatinine (μmol/L)	59.3 (53.4–65.1)	56.7 (51.2–62.2)	0.513

Abbreviations: AUC, area under the curve, BE, base excess; EEG/aEEG, amplitude-integrated electroencephalography; IA-HI, inflammation-amplified hypoxia–ischaemia; MABP, mean arterial blood pressure.

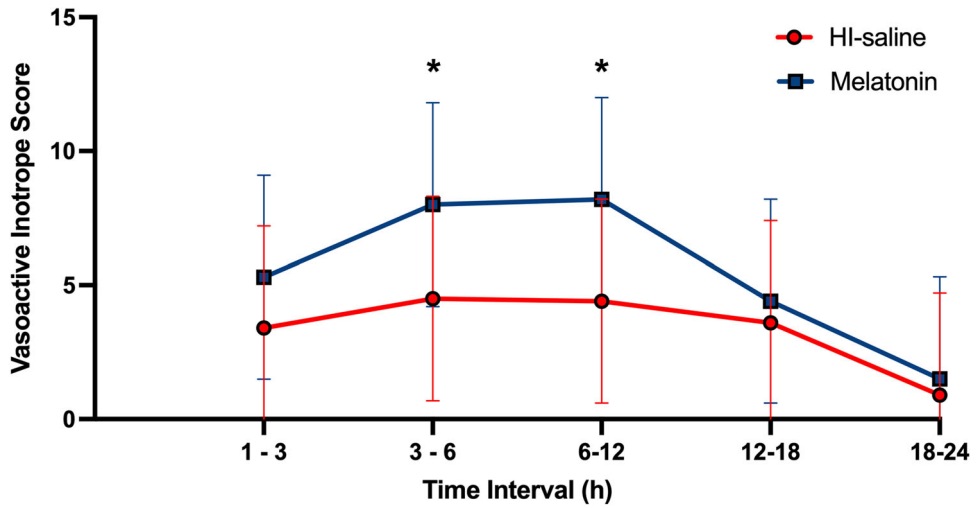

FIGURE 2 Vasoactive Inotrope Score (VIS) between groups to maintain normotension (MABP ≥40 mmHg) (mean ± SD). VIS = dopamine (μg/kg/min) + dobutamine (μg/kg/min) + 100 × adrenaline (μg/kg/min) + 100 × noradrenaline (μg/kg/min), where *$p < .05$ compared to HI-saline.

3.4 | Magnetic resonance spectroscopy

^{1}H and ^{31}P MRS results were available for 22 animals and are shown in Figure 4. One animal in the HI-saline group was excluded due to poor spectral fit.

At 60 h, melatonin was associated with a reduction in BGT Lac/NAA peak area ratio of 0.197 $\log_{10}$ units (95% CrI (−0.366, −0.028)) (see Figure 4D–F). Bayesian analysis showed a $Pr_{(sup)}$ of 98.8% (see Figure 4F). The probability to reduce BGT Lac/NAA by 0.05, 0.1

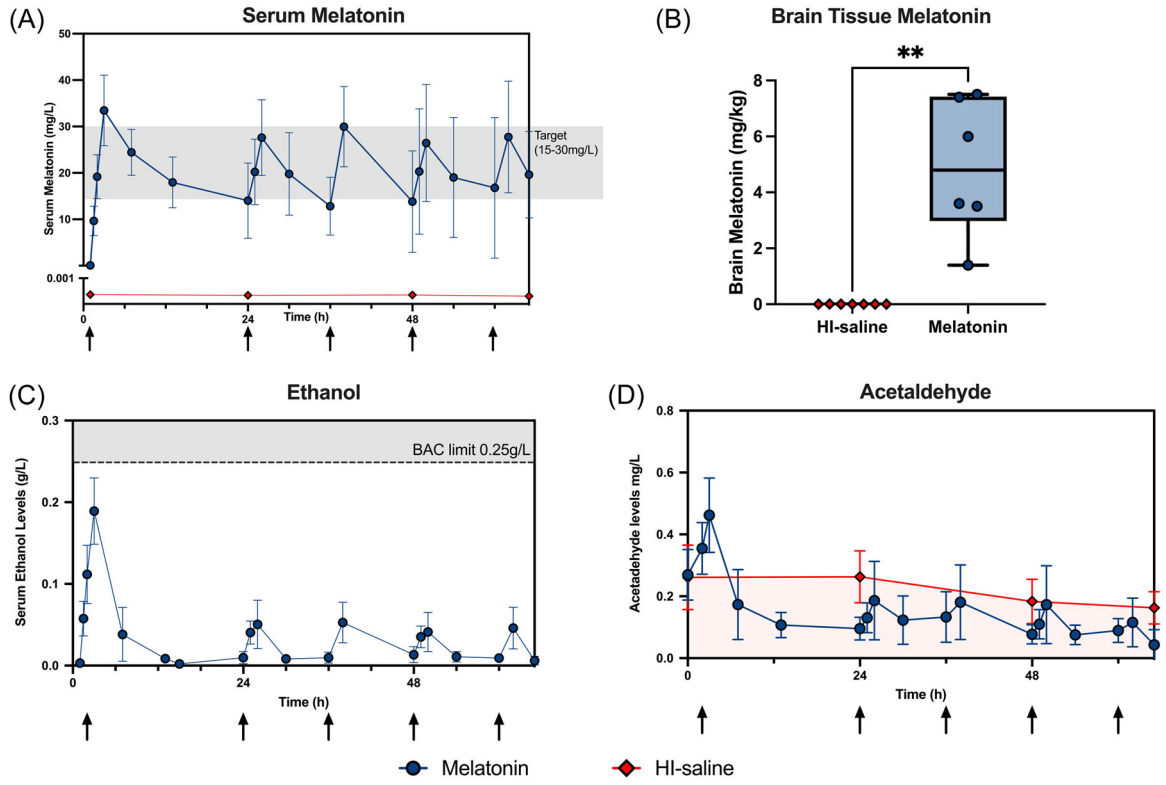

FIGURE 3 Pharmacokinetics. Target serum melatonin levels (mean ± SD) of 15–30 mg/L were achieved within 3 h of HI and maintained throughout the study and were 10^4 higher than HI-saline (A). Brain tissue melatonin levels were 10^3-fold higher in melatonin-treated animals versus saline (B). Serum ethanol levels were below 0.25 g/L through the experiments (C) and with the exception of the loading dose, peak acetaldehyde levels were similar compared to the HI-saline group (D). $^{**}p < .01$.

TABLE 2 Population-based pharmacokinetic parameter estimates for melatonin.

	Basic model (unadjusted)	Full model (adjusted)
Observations	63	63
Number of animals	12	12
Clearance (L/h)	0.076 (0.055–0.106)	0.086 (0.061–0.123)
Volume distribution (L)	1.14 (1.03–1.27)	1.26 (1.1–1.43)
Half-life (h)	10.4	10.2
Residual variability (CV %)	5.56	2.60
Objective function value (OFC)	911	818

Note: PK parameter estimate outputs from the population-based model are presented as mean (95% CI where shown). Adjusted model includes co-variants: age (in hours from IA-HI) and weight (kg) at baseline.

and 0.15 $\log_{10}$ units were 94.4%, 81.6% and 58.2%, respectively. In the WM voxel, the probability of treatment superiority was 89.3% (−0.257, 95% CrI [−0.676, 0.164]) (see Figure 4A–C). On ^{31}P MRS, melatonin was associated with a higher PCr/Epp ratio (+0.041, 95% CrI [0, 0.081] and $Pr_{(sup)}$ of 97.6%) (see Figure 4G–I).

3.5 | aEEG/EEG

aEEG/EEG data were available for all 23 animals and shown in Figure 5. Melatonin was associated with a probability of 86.1% to improve background aEEG/EEG activity. Melatonin was associated with earlier aEEG/EEG recovery from 19 to 24 h (score difference

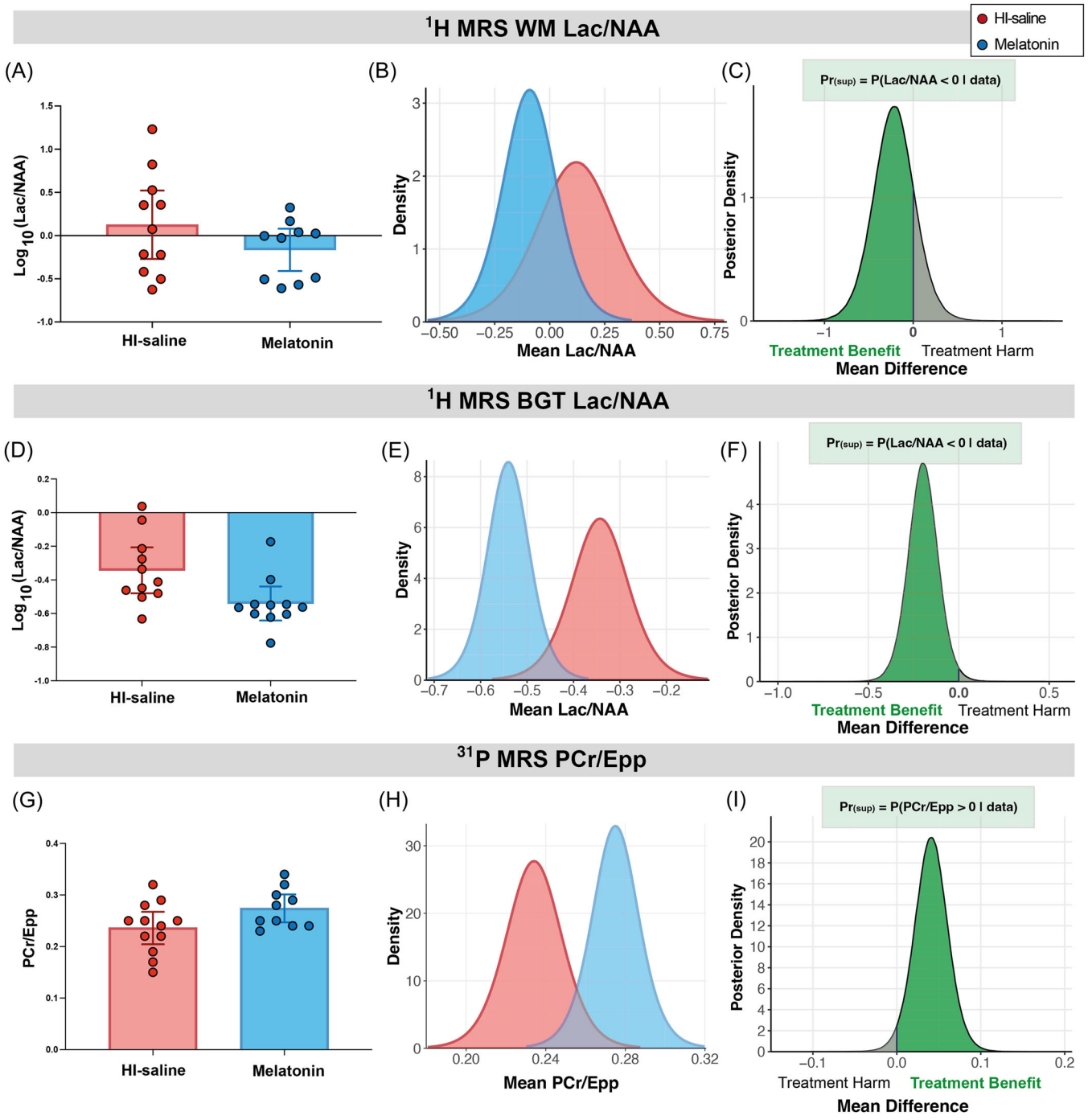

FIGURE 4 Magnetic resonance spectroscopy.[1]H MRS Lac/NAA peak area ratio between melatonin vs HI-saline in the WM voxel (A–C) and BGT voxel (D–F) and [31]P MRS PCr/Epp ratio (G–I) are shown. Data presented as metabolite ratios of individual piglets (dots) with group mean (bar) (±SD) in panels (A, D, G), density plots of the metabolite ratio data distribution in panels (B, E, H). The posterior distribution is shown in panels (C, F, I) from Bayesian analysis using a non-informative prior. The probability of treatment superiority was 89.8, 98.8% and 97.6% for WM Lac/NAA, BGT/NAA and PCr/Epp, respectively. Probability of treatment superiority was defined by posterior probability of Lac/NAA peak area ratio <0 and PCr/Epp ratio >0 in melatonin versus HI-saline.

+0.4 (95% CrI −0.07, 0.86)), with $Pr_{(sup)}$ of 95.4%. From 7 h to 60 h, $Pr_{(sup)}$ ranged from 80% to 95.4% (see Figure 5A).

Five animals in HI-saline (41.7%) versus one animal in the melatonin group (9%) did not recover to at least an aEEG/EEG score of 3. Seizures were detected in five animals (21.7%): four in the HI-saline arm (from 11 to 25 h after IA-HI) and one in the melatonin arm (from 13 h after insult). Four animals required treatment with phenobarbitone (HI-saline

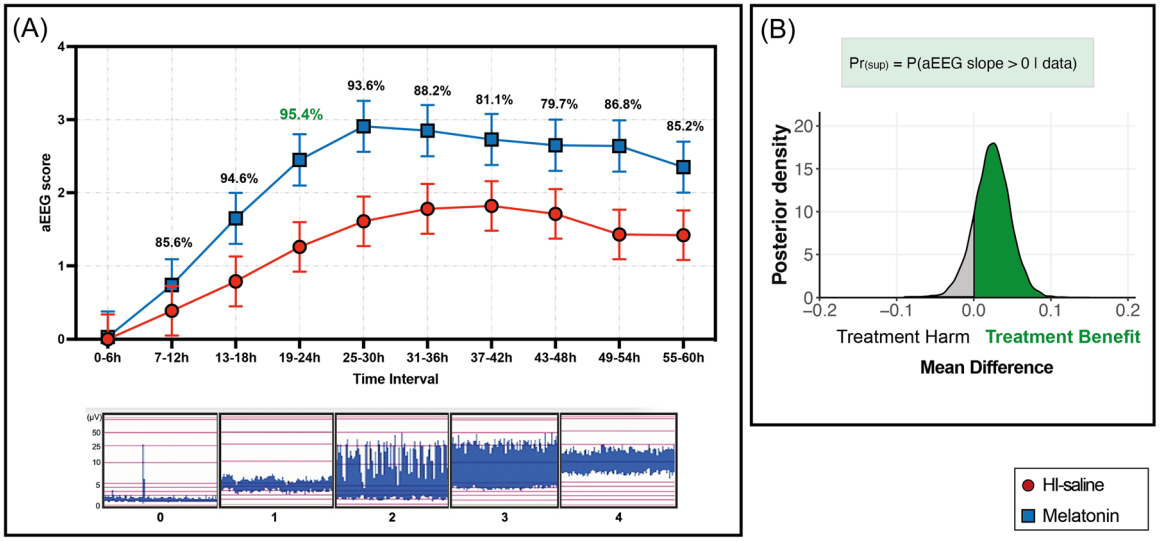

FIGURE 5 Amplitude-integrated encephalography (EEG/aEEG). aEEG scores were classified in accordance with the voltage criteria[29] (see panel), averaged over 6 h intervals and compared (mean ± SEM). Melatonin-treated animals showed early EEG/aEEG recovery at 19–24 h (*probability of superiority of 95.4%). Probability of treatment superiority was defined by posterior probability of aEEG slope >0 in melatonin versus HI-saline. Box showing example micrographs.

$n = 3$, melatonin $n = 1$) and one terminated spontaneously without treatment (HI-saline).

3.6 | Immunohistochemistry

Brain immunohistochemistry was assessed for TUNEL-positive cells (cells/mm^2), NeuN (cells/mm^2), Iba-1 (cells/mm^2), OLIG2 (cells/mm^2) and GFAP (% positive area) and shown in Figure 6.

The probability of melatonin reducing the overall TUNEL-positive cell count was 89.7% (mean difference −0.28, 95% CrI (−0.71, 0.18) (see Figure 6A). Regional analysis showed a significant reduction in TUNEL-positive counts in the hippocampus (−0.81, 95% CrI (−1.48, 0.15), $Pr_{(sup)}$ 99.2%) with promising reduction also seen in the internal capsule and putamen ($Pr_{(sup)}$ 94.2% and 93.2% respectively) (Figure 6B).

Melatonin was associated with an overall increase in NeuN-positive cells ($Pr_{(sup)}$ 98.8%) (mean −2.65, 95% CrI [0.63, 4.76]) (Figure 6C). We observed a significant increase in NeuN+ cell density in the cingulate and sensorimotor cortices, hippocampus, caudate and putamen ($Pr_{(sup)}$ 99.2%, 98.2%, 98.6%, 99.7% and 99.3%, respectively) (see Figure 6D).

The probability of melatonin reducing the overall Iba-1-positive cells was 84% (mean −1.2, 95% CrI [−3.85, 1.25]) (see Figure 6E). Regional analysis showed a significant reduction in IBA1-positive cells in the putamen (−3.52, 95% CrI [−6.8, −0.53], $Pr_{(sup)}$ 98.8%) with promising reduction observed in the thalamus ($Pr_{(sup)}$ 92.9%) (see Figure 6F).

We observed no significant difference in the overall or regional density of % GFAP positivity or OLIG2+ve cell counts (see Figure 6G–I).

3.7 | Peripheral markers of inflammation

We observed no significant difference in the total white cell, platelet, neutrophil and lymphocyte counts between melatonin and HI-saline-treated animals (see Figure 7A–D). SIRI and SII (see Figure 7E,F) reduced over time ($p < .05$), however, did not differ significantly between the groups at all time points. Plasma pro- (IL-6) and anti-inflammatory cytokines (IL-1ra, IL-4, IL-10, IL-12) reduced over the time from HI ($p < .001$) (see Figure 7G–L). We observed no significant differences in cytokines between the groups except lower IL-10 levels in melatonin-treated animals at 48 h (LS mean difference 0.87, 95% CI 0.14–1.59, $p = .019$) (see Figure 6J).

4 | DISCUSSION

In this newborn piglet study relevant to the low-resource setting, intravenous melatonin monotherapy, commenced at 1 h after IA-HI, demonstrated treatment efficacy across neurometabolic (MRS), neurophysiological (aEEG/EEG) and neuropathological (immunohistochemistry) outcome measures. Findings from this study add to the body of evidence[11]

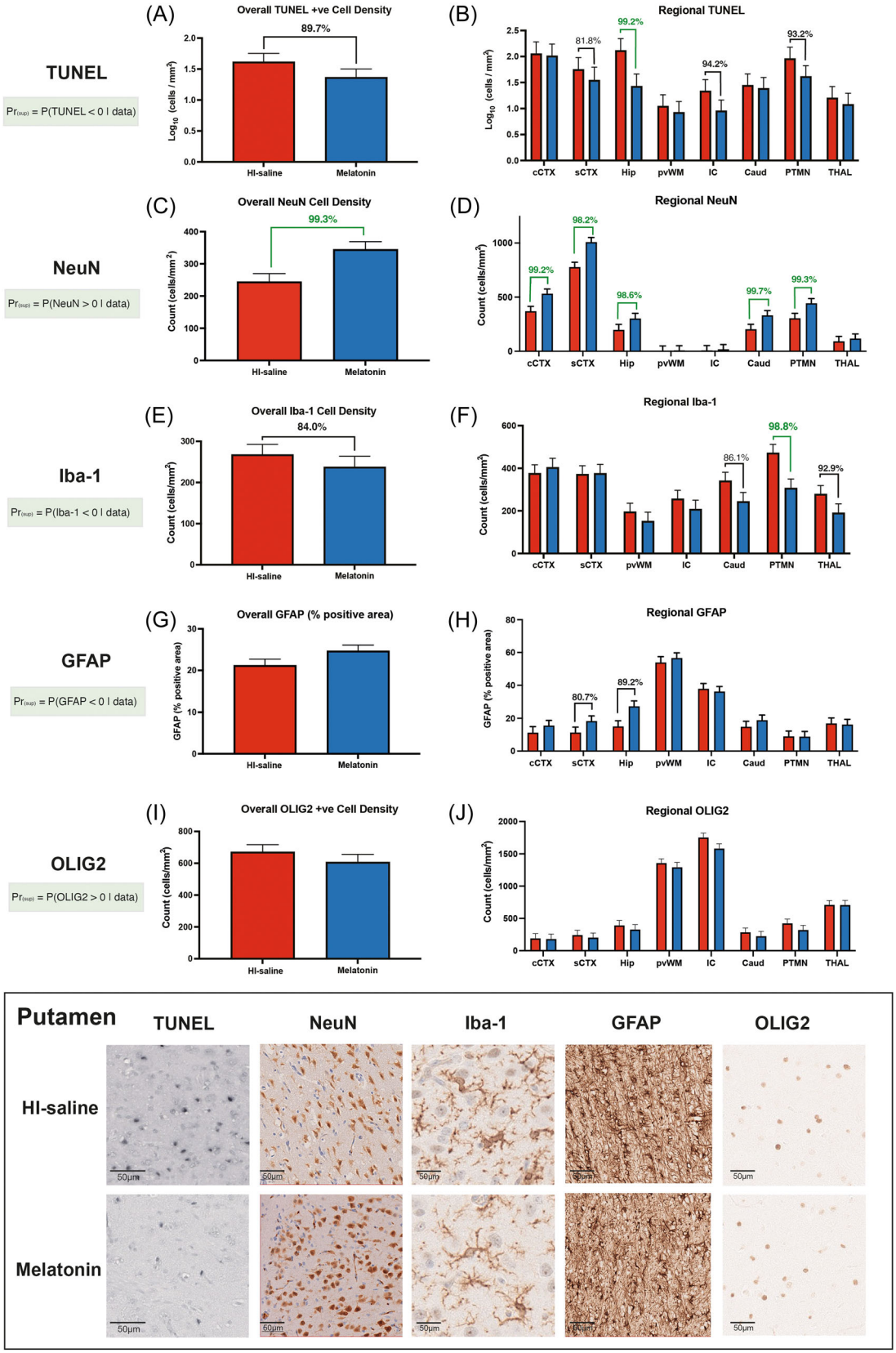

FIGURE 6 (See caption on next page).

supporting translation of melatonin to early-phase clinical trials in babies with NE.

This study provided further evidence of the strong safety profile of high-dose, intravenous melatonin administration. We observed no significant differences in the physiological and biochemical parameters between melatonin and HI-saline animals. The inotropic support requirements were marginally higher in melatonin-treated animals in the first 3–12 h following HI (increase in dopamine of ~3 μg/kg/min). The implications of this marginal increase in inotropic requirements is unclear; however, it highlights the need to study melatonin in a phase I dose escalation study in high-income settings where babies have continuous invasive blood pressure measurement, starting at a quarter (5 mg/kg) dose, increasing incrementally while carefully assessing the effect on blood pressure and inotrope requirement. A phase I safety study will need to be repeated in LMICs. In this optimised melatonin administration regimen, the loading dose of 20 mg/kg achieved therapeutic target levels rapidly within 3 h of IA-HI. Subsequent 12-hourly maintenance doses of 10 mg/kg ensured levels of 15–30 mg/L were maintained. This therapeutic target was established in previous studies in piglets and is summarised in Figure 8.[34] In this current study of IA-HI, where cooling is not protective,[6,9,35] melatonin monotherapy targeting this therapeutic range is cytoprotective, highlighting its diverse benefits. Aridas and colleagues[13] observed improvement in Lac/NAA, histology and neurobehavioural outcomes with melatonin doses of ~15 mg/kg/day. While serum melatonin levels were 10-fold lower than our target range, melatonin administration was earlier (at 30 min) and given as multiple 2-hourly 5 mg boluses. Lower melatonin levels may suffice if melatonin is administered earlier following HI; however, our 2-h infusion every 12–24 h is more feasible in the clinical setting. The anticipated delay between birth and recruitment to clinical trials necessitates rapid attainment of therapeutic levels with a loading dose.

The population-PK parameters following intravenous melatonin administration are not well characterised in the target population of term infants with NE. The similarities between pigs and humans in anatomy and physiology, including drug metabolising enzymes, suggest pigs are well suited to predicting the PK profile in humans.[36] Melatonin is metabolised by the hepatic CYP450 system[37] and renally cleared,[38] processes which may be affected by the systemic HI injury. The volume of drug distribution in this *term*-equivalent piglet study was similar to that in a study of intravenous melatonin administration to preterm infants by Merchant et al.,[39] although the half-life in piglets (10.2 h) was shorter than preterm babies (16.9 h). In term infants, PK data are limited to a study by Balduini et al.,[40] who reported a prolonged half-life of 26 h in cooled infants with HIE following *oral* melatonin.[39] The melatonin half-life in babies and our piglets was significantly prolonged compared to the adult half-life of 30–60 min.[41]

An important consideration for clinical translation is limiting the risk associated with excipient use. Our preclinical meta-analysis suggested ethanol-containing formulations may provide improved protection.[11] This is supported by the partial benefit of low-dose ethanol observed in newborn piglets[12] and foetal sheep[25] following HI. The concerns with chronic alcohol exposure during foetal development and developmental impairment likely relate to the chronicity of exposure and BAC, with a U-shaped dose–response relationship suggested. In preclinical adult stroke models, ethanol doses of 1.5 g/kg resulting in BAC of 0.89 g/L[42–44] were protective, but a higher dose of 3 g/kg (BAC 2 g/L) was toxic.[45] In preclinical studies of the immature brain, toxicity was observed at a lower BAC threshold of 1 g/L,[46–48] highlighting increased vulnerability of the developing brain. Ethanol-containing medicines are already commonplace in drugs prescribed in neonatal units including furosemide, iron and phenobarbital.[49] In this study, the ethanol dose (320 mg/kg) was comparable to the partially protective dose observed in our previous piglet neuroprotection study (285 mg/kg). Importantly, the loading dose resulted in BAC below the American Academy of Paediatrics[26] advisory limit of 0.25 g/L and less than a quarter of toxic levels in rodents discussed earlier.[46–48] We limited the accumulation of ethanol by reducing the infusion rate of the maintenance doses to below the rate of ethanol metabolism (0.1 g/kg/h[50]). The BAC levels were low and similar to the exposure associated with furosemide administration in preterm infants (0–73 mg/L),[51] and

FIGURE 6 Immunohistochemistry. Eight regions were assessed for TUNEL-positive cell density (A, B), NeuN+ve cell density (C, D), Iba-1 cell density (7 regions) (E, F), GFAP % positive area (G, H) and OLIG2+ve cell density (I, J). Data shown as overall and regional means. The probability of treatment superiority is shown where PCr > 80% with Pr > 94.8% (superiority threshold) in green. cCTX, cingulate gyrus; CAUD, caudate nucleus; HIP, hippocampus; IC, internal capsule; PvWM, periventricular white matter; PTMN, putamen; sCTX, sensorimotor cortex; THAL, thalamus. Probability of treatment superiority was defined by posterior probability of count <0 in TUNEL, Iba-1 and GFAP and count >0 in NeuN and OLIG2 with melatonin versus HI-saline. Example micrograph shown from putamen (see box).

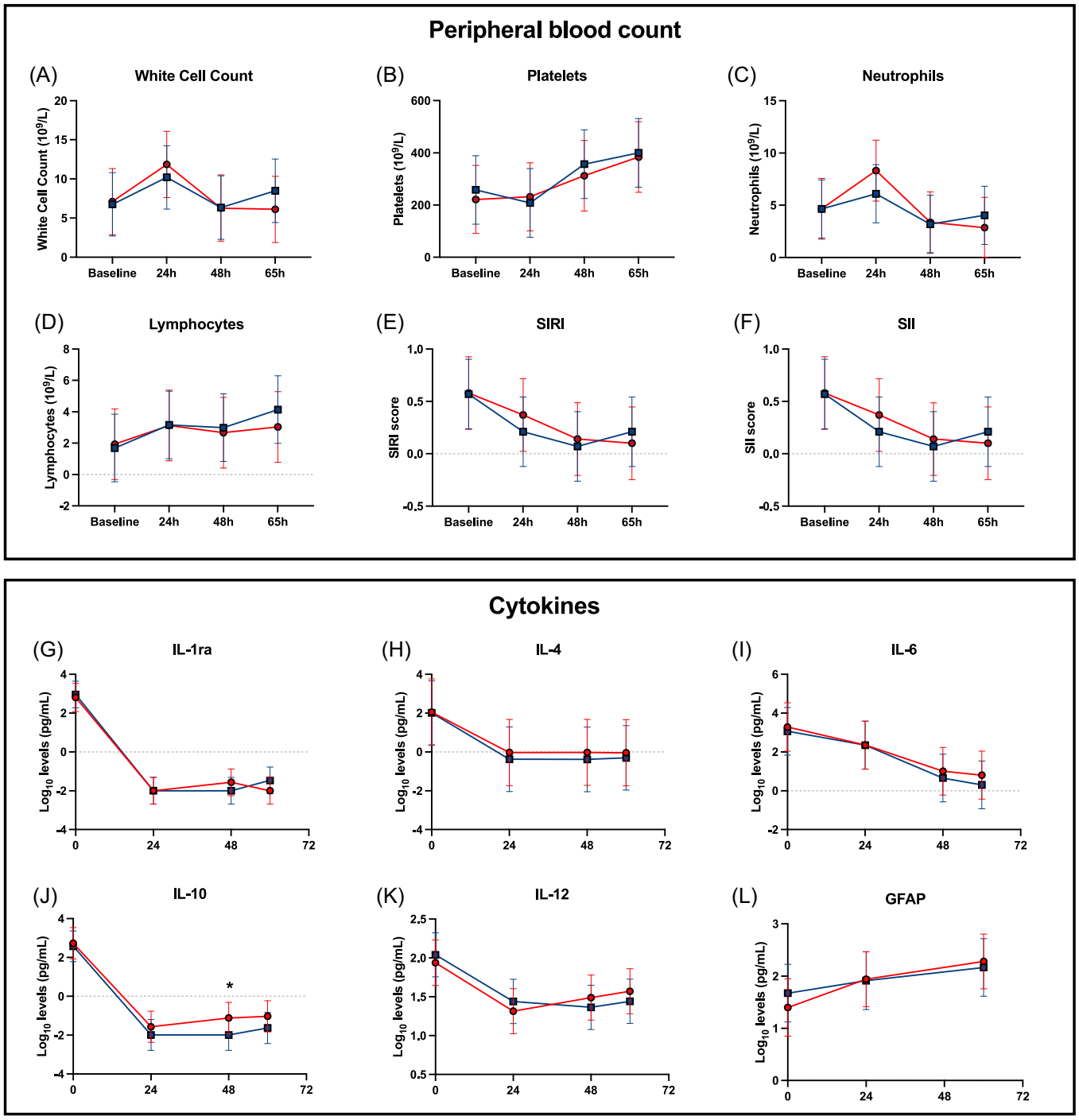

FIGURE 7 Peripheral Blood Markers of Inflammation. Plasma samples were measured for blood counts including total white cells (A), platelets (B), neutrophils (C) and lymphocytes (D). The systemic inflammation response index (SIRI) and systemic immune-inflammation index (SII) were deduced. Plasma cytokines including IL-1ra (G), IL-4 (H), IL-6 (I), IL-10 (J), IL-12 (K) and GFAP (L) were measured at 0 (end of HI), 24, 48 and 60 h. Data presented as LS means ± SD where $*p < .05$ in melatonin versus HI-saline (ANOVA analysis).

other medicines in the NICU.[52] While the by-product of ethanol metabolism, acetaldehyde, may contribute to ethanol-induced toxicity,[53] levels were not significantly higher than those in preterms prescribed iron and furosemide (range 0–9 mg/L).[51] Acetaldehyde did not accumulate following the maintenance doses and was comparable to levels in preterm infants without ethanol exposure (0–0.14 mg/L).[51]

Melatonin was associated with improvement in clinically important surrogate biomarkers of neonatal

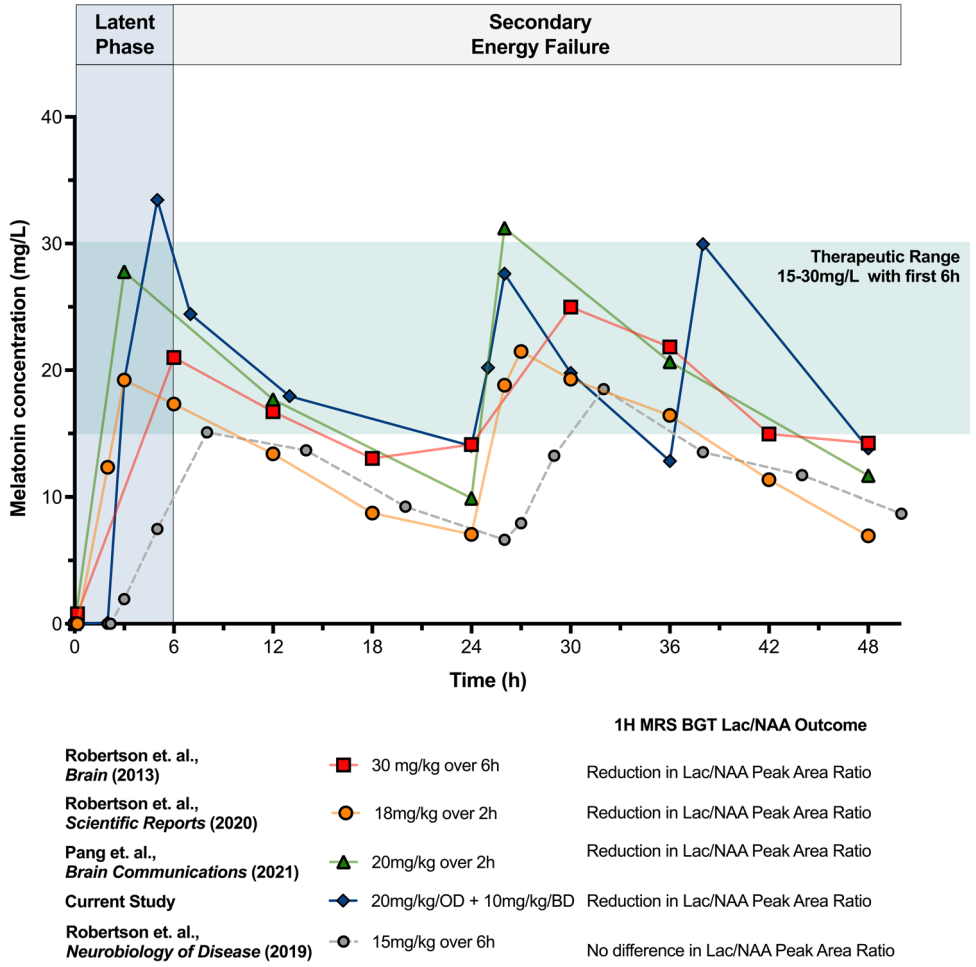

FIGURE 8 Summary of melatonin PK profile in newborn piglet studies.[12,18,19,28] Attainment of serum melatonin levels of 15–30 mg/L within the latent phase of injury (<6 h after HI) is necessary for reduction in Lac/NAA.

outcomes. Lac/NAA peak area ratio predicts 2-year neurodevelopmental outcomes in babies with NE with high accuracy[54] and has been validated as an early quantitative read-out for use in neonatal neuroprotection trials.[55] In piglets, Lac/NAA correlates closely with immunohistochemistry markers of cell death and neuro-inflammation.[56] Extrapolating from the logistic regression model comparing Lac/NAA to outcomes in babies with moderate to severe NE published in Mitra et al.,[54] the observed BGT Lac/NAA reduction of 0.23 $\log_{10}$ units in melatonin versus HI-saline in this study translates to a 87%, 50% and 34% improvement in motor, cognitive and language outcomes, respectively. These are important outcomes as ~40% of infants develop adverse cognitive outcomes.[57] We observed a reduction in WM Lac/NAA despite the reduced statistical power secondary to the large variance from CSF partial volume effect. Improved neurophysiological recovery on aEEG/EEG at 18–24 h was consistent with previous piglet studies of melatonin with cooling.[12,19] aEEG/EEG recovery correlates closely with improved outcomes in infants treated with and without HT.[58–60] Over half of the HI-saline animals did not recover to at least a discontinuous normal voltage (normal trace) background pattern compared to only one piglet in the melatonin group. In a clinical study by Thoresen, infants who never regained a normal background trace within 72 h of birth had poor outcomes compared all infants who recovered by 24 h having good outcomes.[58] The potential for melatonin to improve EEG activity within this critical time interval provides promise that this may translate to improvement in clinically important outcomes. The strong benefit of melatonin on Lac/NAA and aEEG/EEG is complemented by the improvement in NeuN+ cell density suggesting improved neuronal survival. Interestingly, we observed a more modest reduction in TUNEL-positive cell death. TUNEL is a nonspecific marker of several cell death pathways, including apoptosis, necrosis and autolysis.[61] TUNEL-negative cell death pathways have been reported[62] and cells

can escape TUNEL staining, underestimating the population of cell death, particularly where several pathways co-exist.[63]

Surprisingly, we observed a limited biological effect of melatonin on neuro-inflammation in this study.[16,64,65] Melatonin was associated with a reduction in Iba-1 positive cells in the deep grey matter but we observed no difference in circulating plasma cytokine levels or differential full blood count. As Iba-1 is a pan-microglia marker, characterising the balance between the M1 pro-inflammatory versus M2 anti-inflammatory phenotypes may define the immunomodulatory properties of melatonin more clearly, as in a recent preclinical adult stroke study.[66] A cytokine storm may be triggered in this IA-HI model as plasma IL-6 and IL-10 levels were 100-fold higher compared to a previous HI piglet study.[67] These excessively high cytokine levels, leading to overwhelming immune hyperactivation, may impair the ability of melatonin to modulate the immune response. Indeed, O'Dea et al. reported relative immune hyporesponsiveness to endotoxin challenge among infants with NE with elevated cytokine levels.[68] Additional therapies targeting immunomodulation such as azithromycin[69,70] may complement the neuroprotective effect of melatonin.

There are limitations to our study. First, while both sexes were included, the study was not powered to assess the sexual dimorphic response. The study was designed to assess the effect of melatonin compared to HI alone and a control group of non-injured animals was not included. We are therefore unable to determine whether the observed protection is partial compared the non-injured animals. This was a pragmatic decision based on the need to limit animal use in preclinical research. Previous studies in foetal sheep demonstrated complete protection following HI on MRS and immunohistochemistry with melatonin administration to levels similar to non-injured animals.[13,16,17] Interestingly, Malhotra et al.[71] observed an over-abundance of myelin basic protein and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) in growth-restricted foetal sheep following maternal melatonin administration compared to non-injured animals. This provided insight into the potential promotion of myelination and oligodendrocyte maturation associated with melatonin, also reported by other groups.[72] Neurobehavioural outcomes were not assessed in this study; however, Lac/NAA and aEEG/EEG are clinical surrogate biomarkers that accurately predict 2-year outcomes in infants with NE. Neurobehavioural assessment of piglets may not translate to neurodevelopmental outcomes in humans and not without risks, including intractable seizures and aspiration pneumonia.[73] NE is a heterogeneous disease and inflammation may not be the only contributing factor in LMICs. A partial prolonged HI model may better mimic the situation in low-resource settings, given the observed high frequency of seizures in the HELIX trial[3]; however, such a model may necessitate a large sample size.[74]

The decision to translate therapies to early-phase trials requires confirmation of safety and benefit in multiple preclinical models. Indeed, over the last decade, melatonin has demonstrated protection on multiple outcome measures in large and small animal models of HI[11]. In this newborn piglet of IA-HI, under circumstances where HT is not protective, intravenous melatonin administration (achieving serum melatonin of 15–30 mg/L within 3 h) was associated with robust cytoprotection across neurometabolic, neurophysiological and histological outcomes. No significant physiological safety concerns were observed with this optimised melatonin (with ethanol excipient) and BAC was below safety limits. Overall, the preclinical evidence for melatonin as a monotherapy in babies with NE in LMICs is robust, supporting clinical translation.

AUTHOR CONTRIBUTIONS

Raymand Pang, Christopher Meehan and Nicola J. Robertson conceptualised and designed the study with input from John Barks. Raymand Pang, Christopher Meehan, George Maple, Georgina Norris, Ellie Campbell, Katie Tucker and Alison Mintoft undertook the experiments and collected the data. Francisco Torrealdea and Alan Bainbridge acquired the MRS data with support from Xavier Golay. Christopher Meehan and Mariya Hristova performed the immunohistochemistry analysis. Raymand Pang and Joseph Standing performed the pharmacokinetics analysis. Raymand Pang and Nicola J. Robertson performed the data analysis and interpretation. Raymand Pang prepared the first draft of the manuscript. All authors critically reviewed and approved the article.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Lee AC, Kozuki N, Blencowe H, et al. Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. *Pediatr Res*. 2013;74(suppl 1):50-72. doi:10.1038/pr.2013.206
2. Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. Cooling for newborns with hypoxic ischaemic encephalopathy. *Cochrane Database Syst Rev*. 2013;(1):CD003311. doi:10.1002/14651858.CD003311.pub3
3. Thayyil S, Pant S, Montaldo P, et al. Hypothermia for moderate or severe neonatal encephalopathy in low-income and middle-income countries (HELIX): a randomised controlled trial in India, Sri Lanka, and Bangladesh. *Lancet Glob Health*. 2021;9(9):e1273-e1285. doi:10.1016/S2214-109X(21)00264-3
4. Tann CJ, Nakakeeto M, Willey BA, et al. Perinatal risk factors for neonatal encephalopathy: an unmatched case-control study. *Arch Dis Child Fetal Neonat Ed*. 2018;103(3):F250-F256. doi:10.1136/archdischild-2017-312744
5. Martinello KA, Meehan C, Avdic-Belltheus A, et al. Acute LPS sensitization and continuous infusion exacerbates hypoxic brain injury in a piglet model of neonatal encephalopathy. *Sci Rep*. 2019;9(1):10184. doi:10.1038/s41598-019-46488-y
6. Martinello KA, Meehan C, Avdic-Belltheus A, et al. Hypothermia is not therapeutic in a neonatal piglet model of inflammation-sensitized hypoxia-ischemia. *Pediatr Res*. 2021;91:1416-1427. doi:10.1038/s41390-021-01584-6
7. Eklind S, Mallard C, Arvidsson P, Hagberg H. Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. *Pediatr Res*. 2005;58(1):112-116. doi:10.1203/01.PDR.0000163513.03619.8D
8. Eklind S, Mallard C, Leverin AL, et al. Bacterial endotoxin sensitizes the immature brain to hypoxic--ischaemic injury. *Eur J Neurosci*. 2001;13(6):1101-1106. doi:10.1046/j.0953-816x.2001.01474.x
9. Andersen M, Andersen HB, Andelius TCK, et al. No neuroprotective effect of therapeutic hypothermia following lipopolysaccharide-sensitized hypoxia-ischemia: a newborn piglet study. *Front Pediatr*. 2023;11:1268237. doi:10.3389/fped.2023.1268237
10. Falck M, Osredkar D, Maes E, et al. Hypothermic neuronal rescue from infection-sensitised hypoxic-ischaemic brain injury is pathogen dependent. *Dev Neurosci*. 2017;39(1-4):238-247. doi:10.1159/000455838
11. Pang R, Han HJ, Meehan C, Golay X, Miller SL, Robertson NJ. Efficacy of melatonin in term neonatal models of perinatal hypoxia-ischaemia. *Ann Clin Transl Neurol*. 2022;9:795-809. doi:10.1002/acn3.51559
12. Robertson NJ, Lingam I, Meehan C, et al. High-dose melatonin and ethanol excipient combined with therapeutic hypothermia in a newborn piglet asphyxia model. *Sci Rep*. 2020;10(1):3898. doi:10.1038/s41598-020-60858-x
13. Aridas JD, Yawno T, Sutherland AE, et al. Melatonin augments the neuroprotective effects of hypothermia in lambs following perinatal asphyxia. *J Pineal Res*. 2021;71:e12744. doi:10.1111/jpi.12744
14. Cetinkaya M, Alkan T, Ozyener F, Kafa IM, Kurt MA, Koksal N. Possible neuroprotective effects of magnesium sulfate and melatonin as both pre- and post-treatment in a neonatal hypoxic-ischemic rat model. *Neonatology*. 2011;99(4):302-310. doi:10.1159/000320643
15. Ozyener F, Çetinkaya M, Alkan T, et al. Neuroprotective effects of melatonin administered alone or in combination with topiramate in neonatal hypoxic-ischemic rat model. *Restor Neurol Neurosci*. 2012;30(5):435-444. doi:10.3233/RNN-2012-120217
16. Aridas JDS, Yawno T, Sutherland AE, et al. Systemic and transdermal melatonin administration prevents neuropathology in response to perinatal asphyxia in newborn lambs. *J Pineal Res*. 2018;64(4):e12479. doi:10.1111/jpi.12479
17. Yawno T, Castillo-Melendez M, Jenkin G, Wallace EM, Walker DW, Miller SL. Mechanisms of melatonin-induced protection in the brain of late gestation fetal sheep in response to hypoxia. *Dev Neurosci*. 2012;34(6):543-551. doi:10.1159/000346323
18. Robertson NJ, Martinello K, Lingam I, et al. Melatonin as an adjunct to therapeutic hypothermia in a piglet model of neonatal encephalopathy: a translational study. *Neurobiol Dis*. 2019;121:240-251. doi:10.1016/j.nbd.2018.10.004
19. Pang R, Avdic-Belltheus A, Meehan C, et al. Melatonin and/or erythropoietin combined with hypothermia in a piglet model of perinatal asphyxia. *Brain Commun*. 2021;3(1):fcaa211. doi:10.1093/braincomms/fcaa211
20. Carloni S, Perrone S, Buonocore G, Longini M, Proietti F, Balduini W. Melatonin protects from the long-term consequences of a neonatal hypoxic-ischemic brain injury in rats. *J Pineal Res*. 2008;44(2):157-164. doi:10.1111/j.1600-079X.2007.00503.x
21. Berger HR, Nyman AKG, Morken TS, Widerøe M. Transient effect of melatonin treatment after neonatal hypoxic-ischemic brain injury in rats. *PLoS One*. 2019;14(12):e0225788. doi:10.1371/journal.pone.0225788
22. Revuelta M, Arteaga O, Alvarez A, Martinez-Ibargüen A, Hilario E. Characterization of gene expression in the rat brainstem after neonatal Hypoxic-Ischemic injury and antioxidant treatment. *Mol Neurobiol*. 2017;54(2):1129-1143. doi:10.1007/s12035-016-9724-6
23. Alonso-Alconada D, Alvarez A, Lacalle J, Hilario E. Histological study of the protective effect of melatonin on neural cells after neonatal hypoxia-ischemia. *Histol. Histopathol*. 2012;27(6):771-783. doi:10.14670/HH-27.771
24. Sun Y, Ma L, Jin M, Zheng Y, Wang D, Ni H. Effects of melatonin on neurobehavior and cognition in a cerebral palsy model of plppr5-/- mice. *Front Endocrinol*. 2021;12:598788. doi:10.3389/fendo.2021.598788
25. Drury PP, Davidson JO, Bennet L, et al. Partial neural protection with prophylactic low-dose melatonin after

asphyxia in preterm fetal sheep. *J Cereb Blood Flow Metab*. 2014;34(1):126-135. doi:10.1038/jcbfm.2013.174

26. Ethanol in liquid preparations intended for children. *Pediatrics*. 1984;73(3):405-407.
27. Robertson NJ, Meehan C, Martinello KA, et al. Human umbilical cord mesenchymal stromal cells as an adjunct therapy with therapeutic hypothermia in a piglet model of perinatal asphyxia. *Cytotherapy*. 2021;23:521-535. doi:10.1016/j.jcyt.2020.10.005
28. Robertson NJ, Faulkner S, Fleiss B, et al. Melatonin augments hypothermic neuroprotection in a perinatal asphyxia model. *Brain*. 2013;136(Pt 1):90-105. doi:10.1093/brain/aws285
29. Hellstrom-Westas L, Rosen I, Svenningsen NW. Predictive value of early continuous amplitude integrated EEG recordings on outcome after severe birth asphyxia in full term infants. *Arch Dis Child Fetal Neonat Ed*. 1995;72(1):F34-F38. doi:10.1136/fn.72.1.f34
30. Pressler RM, Abend NS, Auvin S, et al. Treatment of seizures in the neonate: guidelines and consensus-based recommendations-special report from the ILAE Task Force on neonatal seizures. *Epilepsia*. 2023;64(10):2550-2570. doi:10.1111/epi.17745
31. Laber EB, Shedden K. Statistical significance and the dichotomization of evidence: the relevance of the ASA statement on statistical significance and p-values for statisticians. *J Am Stat Assoc*. 2017;112(519):902-904. doi:10.1080/01621459.2017.1311265
32. Nuzzo R. Scientific method: statistical errors. *Nature*. 2014;506(7487):150-152. doi:10.1038/506150a
33. Ioannidis JPA. What have we (not) learnt from millions of scientific papers with P values? *Am Stat*. 2019;73(suppl 1):20-25. doi:10.1080/00031305.2018.1447512
34. Pang R, Advic-Belltheus A, Meehan C, Fullen DJ, Golay X, Robertson NJ. Melatonin for neonatal encephalopathy: from bench to bedside. *Int J Mol Sci*. 2021;22(11):5481. doi:10.3390/ijms22115481
35. Osredkar D, Thoresen M, Maes E, Flatebø T, Elstad M, Sabir H. Hypothermia is not neuroprotective after infection-sensitized neonatal hypoxic-ischemic brain injury. *Resuscitation*. 2014;85(4):567-572. doi:10.1016/j.resuscitation.2013.12.006
36. Leys K, Stroe MS, Annaert P, et al. Pharmacokinetics during therapeutic hypothermia in neonates: from pathophysiology to translational knowledge and physiologically-based pharmacokinetic (PBPK) modeling. *Expert Opin Drug Metab Toxicol*. 2023;19:461-477. doi:10.1080/17425255.2023.2237412
37. Hardeland R. Melatonin metabolism in the central nervous system. *Curr Neuropharmacol*. 2010;8(3):168-181. doi:10.2174/157015910792246244
38. van der Veen A, Minović I, van Faassen M, et al. Urinary excretion of 6-sulfatoxymelatonin, the main metabolite of melatonin, and mortality in stable outpatient renal transplant recipients. *J Clin Med*. 2020;9(2):525. doi:10.3390/jcm9020525
39. Merchant NM, Azzopardi DV, Hawwa AF, et al. Pharmacokinetics of melatonin in preterm infants. *Br J Clin Pharmacol*. 2013;76(5):725-733. doi:10.1111/bcp.12092
40. Balduini W, Weiss MD, Carloni S, et al. Melatonin pharmacokinetics and dose extrapolation after enteral infusion in neonates subjected to hypothermia. *J Pineal Res*. 2019;66(4):e12565. doi:10.1111/jpi.12565
41. Andersen LPH, Werner MU, Rosenkilde MM, et al. Pharmacokinetics of oral and intravenous melatonin in healthy volunteers. *BMC Pharmacol Toxicol*. 2016;17:8. doi:10.1186/s40360-016-0052-2
42. Wang F, Wang Y, Geng X, et al. Neuroprotective effect of acute ethanol administration in a rat with transient cerebral ischemia. *Stroke*. 2012;43(1):205-210. doi:10.1161/STROKEAHA.111.629576
43. Adam H. Reduced apoptosis by ethanol and its association with PKC-δ and Akt signaling in ischemic stroke. *Aging Dis*. 2014;5(6):366-372. doi:10.14336/AD.2014.0500366
44. Kochanski R, Peng C, Higashida T, et al. Neuroprotection conferred by post-ischemia ethanol therapy in experimental stroke: an inhibitory effect on hyperglycolysis and NADPH oxidase activation. *J Neurochem*. 2013;126(1):113-121. doi:10.1111/jnc.12169
45. Zhao YJ, Yang GY, Ben-Joseph O, Ross BD, Chenevert TL, Domino EF. Acute ethanol effects on focal cerebral ischemia in fasted rats. *Alcohol Clin Exp Res*. 1998;22(3):717-722.
46. Houlé K, Abdi M, Clabough EBD. Acute ethanol exposure during late mouse neurodevelopment results in long-term deficits in memory retrieval, but not in social responsiveness. *Brain Behav*. 2017;7(4):e00636. doi:10.1002/brb3.636
47. Newville J, Valenzuela CF, Li L, Jantzie LL, Cunningham LA. Acute oligodendrocyte loss with persistent white matter injury in a third trimester equivalent mouse model of fetal alcohol spectrum disorder. *Glia*. 2017;65(8):1317-1332. doi:10.1002/glia.23164
48. Dalitz P, Cock M, Harding R, Rees S. Injurious effects of acute ethanol exposure during late gestation on developing white matter in fetal sheep. *Int J Dev Neurosci*. 2008;26(5):391-399. doi:10.1016/j.ijdevneu.2008.03.008
49. Marek E, Kraft WK. Ethanol pharmacokinetics in neonates and infants. *Curr Ther Res*. 2014;76:90-97. doi:10.1016/j.curtheres.2014.09.002
50. li TK. Enzymology of human alcohol metabolism. *Adv Enzymol Relat Areas Mol Biol*. 1977;45:427-483. doi:10.1002/9780470122907.ch6
51. Pandya HC, Mulla H, Hubbard M, et al. Essential medicines containing ethanol elevate blood acetaldehyde concentrations in neonates. *Eur J Pediatr*. 2016;175(6):841-847. doi:10.1007/s00431-016-2714-x
52. Valeur KS, Hertel SA, Lundstrøm KE, Holst H. The cumulative daily tolerance levels of potentially toxic excipients ethanol and propylene glycol are commonly exceeded in neonates and infants. *Basic Clin Pharmacol Toxicol*. 2018;122(5):523-530. doi:10.1111/bcpt.12950
53. Cui J, Liu Y, Chang X, et al. Acetaldehyde induces neurotoxicity in vitro via oxidative stress- and Ca(2+) imbalance-mediated endoplasmic reticulum stress. *Oxid Med Cell Longev*. 2019;2019:1-13. doi:10.1155/2019/2593742
54. Mitra S, Kendall GS, Bainbridge A, et al. 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 Neonat Ed*. 2018;104(4):315478. doi:10.1136/archdischild-2018-315478
55. Azzopardi D, Chew AT, Deierl A, et al. Prospective qualification of early cerebral biomarkers in a randomised trial of

treatment with xenon combined with moderate hypothermia after birth asphyxia. *EBioMed*. 2019;47:484-491. doi:10.1016/j.ebiom.2019.08.034

56. Pang R, Martinello KA, Meehan C, et al. Proton magnetic resonance spectroscopy lactate/N-acetylaspartate within 48 h predicts cell death following varied neuroprotective interventions in a piglet model of hypoxia-ischemia with and without inflammation-sensitization. *Front Neurol*. 2020;11:883. doi:10.3389/fneur.2020.00883
57. Wu YW, Comstock BA, Gonzalez FF, et al. Trial of erythropoietin for hypoxic-ischemic encephalopathy in newborns. *N Engl J Med*. 2022;387(2):148-159. doi:10.1056/NEJMoa2119660
58. Thoresen M, Hellström-Westas L, Liu X, de Vries LS. Effect of hypothermia on amplitude-integrated electroencephalogram in infants with asphyxia. *Pediatrics*. 2010;126(1):e131-e139. doi:10.1542/peds.2009-2938
59. van Rooij LGM, Toet MC, Osredkar D, van Huffelen AC, Groenendaal F, de Vries LS. Recovery of amplitude integrated electroencephalographic background patterns within 24 hours of perinatal asphyxia. *Arch Dis Child Fetal Neonat Ed*. 2005;90(3):F245-f251. doi:10.1136/adc.2004.064964
60. Nyman J, Mikkonen K, Metsäranta M, et al. Poor aEEG background recovery after perinatal hypoxic ischemic encephalopathy predicts postneonatal epilepsy by age 4 years. *Clin Neurophysiol*. 2022;143:116-123. doi:10.1016/j.clinph.2022.09.005
61. Kraupp BG, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology*. 1995;21(5):1465-1468. doi:10.1002/hep.1840210534
62. Chautan M, Chazal G, Cecconi F, Gruss P, Golstein P. Interdigital cell death can occur through a necrotic and caspase-independent pathway. *Curr Biol*. 1999;9(17):967-S1. doi:10.1016/s0960-9822(99)80425-4
63. Kelly KJ, Sandoval RM, Dunn KW, Molitoris BA, Dagher PC. A novel method to determine specificity and sensitivity of the TUNEL reaction in the quantitation of apoptosis. *Am J Physiol Cell Physiol*. 2003;284(5):C1309-C1318. doi:10.1152/ajpcell.00353.2002
64. Balduini W, Carloni S, Perrone S, et al. The use of melatonin in hypoxic-ischemic brain damage: an experimental study. *J Matern Fet Neonat Med*. 2012;25(suppl 1):119-124. doi:10.3109/14767058.2012.663232
65. Sinha B, Wu Q, Li W, et al. Protection of melatonin in experimental models of newborn hypoxic-ischemic brain injury through MT1 receptor. *J Pineal Res*. 2018;64(1):e12443. doi:10.1111/jpi.12443
66. Suofu Y, Jauhari A, Nirmala ES, et al. Neuronal melatonin type 1 receptor overexpression promotes M2 microglia polarization in cerebral ischemia/reperfusion-induced injury. *Neurosci Lett*. 2023;795:137043. doi:10.1016/j.neulet.2022.137043
67. Rocha-Ferreira E, Kelen D, Faulkner S, et al. Systemic pro-inflammatory cytokine status following therapeutic hypothermia in a piglet hypoxia-ischemia model. *J Neuroinflammation*. 2017;14(1):44. doi:10.1186/s12974-017-0821-x
68. O'Dea MI, Kelly LA, McKenna E, et al. Altered cytokine endotoxin responses in neonatal encephalopathy predict MRI outcomes. *Front Pediatr*. 2021;9:734540. doi:10.3389/fped.2021.734540
69. Barks JDE, Liu Y, Wang L, Pai MP, Silverstein FS. Repurposing azithromycin for neonatal neuroprotection. *Pediatr Res*. 2019;86(4):444-451. doi:10.1038/s41390-019-0408-6
70. Barks JDE, Liu Y, Dopp IA, Silverstein FS. Azithromycin reduces inflammation-amplified hypoxic-ischemic brain injury in neonatal rats. *Pediatr Res*. 2021;92:415-423. doi:10.1038/s41390-021-01747-5
71. Malhotra A, Rocha AKAA, Yawno T, et al. Neuroprotective effects of maternal melatonin administration in early-onset placental insufficiency and fetal growth restriction. *Pediatr Res*. 2024. doi:10.1038/s41390-024-03027-4
72. Olivier P, Fontaine RH, Loron G, et al. Melatonin promotes oligodendroglial maturation of injured white matter in neonatal rats. *PLoS One*. 2009;4(9):e7128. doi:10.1371/journal.pone.0007128
73. Thoresen M, Haaland K, Løberg EM, et al. A piglet survival model of posthypoxic encephalopathy. *Pediatr Res*. 1996;40(5):738-748. doi:10.1203/00006450-199611000-00014
74. De Haan HH, Gunn AJ, Williams CE, Gluckman PD. Brief repeated umbilical cord occlusions cause sustained cytotoxic cerebral edema and focal infarcts in near-term fetal lambs. *Pediatr Res*. 1997;41(1):96-104. doi:10.1203/00006450-199701000-00015

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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