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Systemic and transdermal melatonin administration prevents neuropathology in response to perinatal asphyxia in newborn lambs

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Abstract

Perinatal asphyxia remains a principal cause of infant mortality and long-term neurological morbidity, particularly in low-resource countries. No neuroprotective interventions are currently available. Melatonin (MLT), a potent antioxidant, anti-inflammatory and antiapoptotic agent, offers promise as an intravenous (IV) or transdermal therapy to protect the brain. We aimed to determine the effect of melatonin (IV or transdermal patch) on neuropathology in a lamb model of perinatal asphyxia. Asphyxia was induced in newborn lambs via umbilical cord occlusion at birth. Animals were randomly allocated to melatonin commencing 30 minutes after birth (60 mg in 24 hours; IV or transdermal patch). Brain magnetic resonance spectroscopy (MRS) was undertaken at 12 and 72 hours. Animals (control n = 9; control+MLT n = 6; asphyxia n = 16; asphyxia+MLT [IV n = 14; patch n = 4]) were euthanised at 72 hours, and cerebrospinal fluid (CSF) and brains were collected for analysis. Asphyxia resulted in severe acidosis (pH 6.9 ± 0.0; lactate 9 ± 2 mmol/L) and altered determinants of encephalopathy. MRS lactate:N-acetyl aspartate ratio was 2.5-fold higher in asphyxia lambs compared with controls at 12 hours and 3-fold higher at 72 hours ($P < .05$). Melatonin prevented this rise (3.5-fold reduced vs asphyxia; $P = .02$). Asphyxia significantly increased brain white and grey matter apoptotic cell death (activated caspase-3), lipid peroxidation (4HNE) and neuroinflammation (IBA-1). These changes were significantly mitigated by both IV and patch melatonin. Systemic or transdermal neonatal melatonin administration significantly reduces the neuropathology and encephalopathy signs associated with perinatal asphyxia. A simple melatonin patch, administered soon after birth, may improve outcome in infants affected by asphyxia, especially in low-resource settings.

KEYWORDS
brain injury, cell death, melatonin, neuroprotection, oxidative stress, perinatal asphyxia, radiology, transdermal

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1 | INTRODUCTION

The United Nations Millennium Development Goal 4 aimed to reduce the global under-five mortality rate by two-thirds between 1990 and 2015.[1] While there has been significant progress towards this goal, it remains that, for every day in 2015,[1] 16 000 children aged under 5 died. Forty-five per cent of these deaths were in newborns, with prematurity and intrapartum-related complications the leading cause of death.[1] In particular, perinatal asphyxia remains a significant problem in low-resource countries, where the rate of asphyxia is 10-fold higher (10-20 per 1000 live births) than in high-resource countries.[2] The low neonatal death rate associated with perinatal asphyxia in high-resource countries is attributed to the availability of advanced obstetric and neonatal care, including the use of neonatal hypothermia. Hypothermia therapy provides a significant but modest improvement in mortality and long-term neurological morbidities.[3] However, a lack of evidence, safety concerns and high implementation costs and difficulties have resulted in limited use of neonatal cooling in low- and middle-income countries. Meta-analysis shows that hypothermia in low-resource countries does not reduce mortality[4] and, indeed, may worsen outcomes in the presence of sepsis.[5] Now, in the era of Sustainable Development Goals, it is recognised that "high impact, cost-effective interventions" must be a focus to decrease mortality and improve long-term neurological outcomes.[1]

Following perinatal asphyxia, damage to the developing brain evolves dependent on a number of cell death-mediating pathways, which are responsive to inflammatory and oxidative stress mediators.[6] In particular, the ischaemia-reperfusion that occurs after perinatal asphyxia initiates production of reactive oxygen species (ROS), which in turn is associated with lipid and protein peroxidation, and neuronal damage via apoptotic or necrotic pathways.[7-9] Upregulation of ROS occurs within 30 minutes of asphyxia and may continue for days after reperfusion.[10,11] The fetal and neonatal brain are particularly susceptible to oxidative stress due to poorly developed innate defence systems.[12-14] ROS, and particularly hydroxyl radicals, compromise neuronal and glial cell structure and function by initiating lipid membrane peroxidation.[11] Further, oxidative stress and neuroinflammation are intimately linked. ROS can initiate pro-inflammatory cytokine release and microglial activation, and conversely, microglia can release free radicals and pro-inflammatory cytokines.[7,15] Both oxidative stress and neuroinflammation are early events in the cascade of brain injury in the perinatal brain. Targeting these early mechanisms of brain injury is widely considered a promising neuroprotective strategy.[16]

Melatonin (5-methoxy-*N*-acetyltryptamine; MLT) is produced primarily by the pineal gland at night.[17] It provides circadian and seasonal timing cues and is a potent antioxidant.[18,19] To a lesser extent, melatonin is also produced outside of the pineal gland within the skin, retina, ovaries and gastrointestinal tract and is readily found within the cerebrospinal fluid (CSF), bone marrow and bile.[20-24] Receptor and nonreceptor actions mediate MLT's neuroprotective effects,[25] including defence against oxidative stress, energy metabolism, improved immune function and anti-inflammatory and antiapoptotic actions.[26-32] The antioxidant properties of MLT include the direct scavenging of ROS.[20,27,33,34] MLT is also metabolised into further strong antioxidant molecules,[21,22] and MLT induces an upregulation of the antioxidant enzymes GSHPx, GSH reductase and superoxide dismutase.[20,33,35,36] MLT also prevents apoptotic cell death via stabilisation of mitochondrial function and prevents the release of pro-apoptotic proteins in response to insult.[37,38] In the setting of a potential therapy for newborn babies, MLT has many attributes that make it very attractive as a therapy in the newborn. It readily crosses the blood-brain barrier, its antioxidant and anti-inflammatory effects are achieved quickly, and, even in high concentrations, it has an excellent safety profile.[30,39-42]

We undertook this study to examine the neuroprotective effects of MLT in a large animal (lamb) model of perinatal asphyxia and subsequent hypoxic-ischaemic encephalopathy (HIE). We designed this study with consideration of potential MLT administration that would be applicable for births in low-resource countries. We have previously shown that perinatal asphyxia in term lambs closely mimics the clinical characteristics of human HIE, including significant cell death within the brain and functional encephalopathic symptoms.[43] In this study, our aim was to administer MLT to the neonatal lamb following a severe asphyxic insult at birth, to determine the neuroprotective profile of MLT over 72 hours and finally to examine and compare intravenous (IV) vs transdermal administration of MLT delivery. We hypothesised that a significant increase in circulating and brain MLT concentration would be associated with decreased brain lipid peroxidation and inflammation and neuroprotection.

2 | METHODS

Experimental procedures adhered to the NHMRC guidelines for the care and use of animals for scientific purposes[44] and were approved by Monash Medical Centre Animal Ethics Committee.

2.1 | Surgery

We used a large animal model of perinatal asphyxia, as previously described.[43] Briefly, pregnant Merino cross Border Leicester ewes underwent aseptic surgery for Caesarean section delivery at 139-141 days gestation (term 145-147 days); the study always commenced at 09:00 hour. Anaesthesia was induced by sodium-thiopentone and maintained with 1-2.5%

isoflurane (IsoFlow, Abbott, Doncaster, Vic., Australia). The fetal head remained in utero, while the fetal body was exteriorised to allow insertion of femoral artery and vein catheters for clinical care, MLT administration and continuous physiological recordings (PowerLab SP, ADI Instruments, Bella Vista, NSW, Australia). We began recording blood pressure (BP) and heart rate (HR) immediately after the catheter was inserted. The fetal body was then returned to the amniotic cavity, and 10 minutes of baseline in utero recordings were made prior to the asphyxic insult.

2.2 | Asphyxia and resuscitation

Fetal sheep were randomly allocated, using an opaque envelope system, to control, control+MLT, asphyxia, asphyxia+MLT IV or asphyxia+MLT patches (asphyxia+ MLT-P) groups. While the fetus remained within the uterine cavity, the umbilical cord was exposed. An umbilical cord clamp was placed 50 mm from the fetal abdomen. In control fetuses, the umbilical cord was immediately cut, following clamping, and the lamb promptly delivered and resuscitated. Fetuses undergoing asphyxia remained within the amniotic fluid with continuous monitoring of BP and HR. The cord was cut and the lamb delivered when mean BP decreased between 18 and 20 mm Hg.

We have previously published a detailed description of the immediate resuscitation and maintenance care of control and perinatal asphyxia lambs.[9,43] In brief, all lambs were immediately intubated allowing resuscitation with positive pressure ventilation (NeoPuff, Fisher and Paykel Healthcare, Nunawading, Vic., Australia; 30 cm H_2O positive inspiratory pressure [PIP]; 5-8 cm positive end-expiratory pressure [PEEP]; 10 L/min of room air; 30 breaths/min). If BP and HR did not respond to ventilation, adrenaline (1 mL of 1:10 000) and fluid (normal saline, 20 mL/kg, Baxter Healthcare, Brunswick, Vic., Australia) were administered. Following resuscitation, a Babylog 8000+ ventilator provided ongoing mechanical ventilation (Dräger, Notting Hill, Vic., Australia; volume guarantee 5 mL/kg, PEEP 5-7 cm H_2O, 30 breaths/min). Assisted ventilation was decreased and then ceased when the lamb was spontaneously breathing >50% of the time. Blood sampling was undertaken in utero, during asphyxia and postdelivery to assess blood gas parameters and melatonin concentration.

2.3 | Melatonin

Intravenous MLT (Sigma-Aldrich, Castle Hill, NSW, Australia) was administered to a subset of control and asphyxia lambs. MLT was prepared by dissolving 60 mg MLT in 2 mL of absolute ethanol. Normal saline (40 mL; Baxter Healthcare) was added to the solution, which was then filtered and stored in darkness at 4°C. Intravenous MLT was

administered via the femoral vein catheter in a 3.5 mL (5 mg) bolus and the catheter flushed with saline at 30 minutes after birth and then every 2 hours until 24.5 hours postnatal for a total dose of 60 mg.

A further cohort of asphyxia animals was treated with a commercial over the counter 5 mg MLT transdermal patch (asphyxia+MLT-P; Avazo, Warminster, PA, USA). These patches contain 5 mg MLT (N-(2-(5-methoxyindol-3-yl) ethyl)-acetamide) dissolved in a 35 mm² FT-200 polyethylene patch with acrylic copolymer adhesive. The lamb's abdomen was shaved, and 6 patches were placed on the shaved abdomen to deliver 30 mg MLT at 30 minutes after birth. These patches were replaced with 6 new patches at 12 hours, to provide a total of 60 mg MLT over this period. This treatment regimen was guided by published transdermal MLT administration data showing the time for maximum plasma concentration ranged between 1 and 18 hours and with a half-life of 5-10 hours using doses between 2 and 100 mg.[45-49] Patches were removed at 24.5 hours postbirth.

2.4 | Neurobehavioural milestones

An advantage of using neonatal lambs is that they are precocial and can provide a good understanding of normal and abnormal motor and behavioural function within days after birth.[50] Overt clinical seizures were assessed by clinical team members (AM, MCF, FW, EMW) and defined as repetitive eye movements, "smacking" of the lips, neck arching, "running" leg movements, temperature spikes and apnoeic episodes. This is similar to neonatal seizures as described by Volpe.[51] Clinical seizures, when present, were treated with 20 mg/kg phenobarbitone (Sigma-Aldrich), administered intravenously in saline over 30 minutes.

Lambs were provided with maintenance fluid (10% glucose; 40 ml/kg/d) to maintain glucose levels >3.5 mmol/L until oral formula intake was established. Once alert and extubated, lambs with good physical tone and suckling reflex were offered sheep milk formula (50 mL/kg; Wombaroo Food Products, Glen Osmond, SA, Australia) orally.

2.5 | Magnetic resonance spectroscopy

Lambs were lightly sedated with medetomidine hydrochloride (0.1 mg/kg, Domitor, Pfizer, West Ryde, NSW, Australia), and magnetic resonance spectroscopy (MRS) scans undertaken at 12 and 72 hours after birth in a 3T Siemens Verio scanner (Siemens Healthcare, Bayswater, Vic., Australia), with an 8-channel knee coil utilising a 2 cm³ D voxel in the deep grey matter of the brain. For each metabolite (lactate, choline and n-acetyl-aspartate [NAA]), the relative concentrations were measured by computer algorithm of peak area under the MRS curve. The ratios of the 3 metabolites of interest, (NAA:choline, lactate:choline and lactate:NAA) at 12

and 72 hours were calculated. MRS was not undertaken on asphyxia+MLT-P lambs, but all other neurological outcomes are presented.

2.6 | Biochemical neuropathology markers in cerebrospinal fluid

Melatonin concentration was assessed in plasma (collected in utero and at 4, 12, 24 and 48 hours postdelivery) and brain tissue (cerebral cortex homogenate, collected at post-mortem) by radioimmunoassay, as per manufacturer's instructions (Buhlmann Laboratories AG, Schönenbuch, Switzerland). The intra-assay precision was 7.9%, interassay precision of 11.7% with sensitivity of 0.3 pg/mL. Malondialdehyde (MDA), a marker for oxidative stress, was measured using a thiobarbituric acid-reacting substances assay (Cayman Chemical Company, Ann Arbor, MI, USA) in CSF collected at post-mortem.[50] The assay sensitivity was 0.1 µmol/L, with 5.1% interassay and 3.1% intra-assay co-efficients of variation. S100B, a marker of cellular injury, was measured via ELISA as per manufacturer's instructions (DiaSorin, Stillwater, MN, USA) on CSF. Assay sensitivity was 0.03 µg/L with a run imprecision of <10% and total imprecision of <15%. Flex Set Cytokine Bead Array Kits (BD Biosciences, North Ryde, NSW, Australia) were utilised to determine concentration of cytokines IL-1β, IL-6, IL-10 and tumour necrosis factor-α (TNF-α) in CSF following manufacturer's instructions. There was an >85% recovery of the standards, with intra-assay coefficients of variation for IL-6, 164%; IL-10, 120%; IL1-β, 157%; and TNF-α, 116% and an assay sensitivity of 0.274 pg/mL.

2.7 | Neurohistopathology

Lambs were euthanised (5 mL, pentobarbitone, Lethabarb, Virbac, Milperra, NSW, Australia) after the 72 hours MRS. The brain was removed and weighed. The right cerebral hemisphere was cut coronally into 5-mm slices from the frontal cortex and immersion fixed in 4% paraformaldehyde for 48 hours, paraffin embedded and then sliced at 10 µm for histology. Slides were dewaxed in xylene and rehydrated through a series of ethanol dilutions before histological assessment. Two duplicate slides of each section (5 mm apart) per animal were examined. Within each region of interest, the average number of positive cells was counted across 3 fields of view. Results were then averaged across all animals in each group. The brain regions of interest were the hippocampus (dentate gyrus), midtemporal cortex (pleomorphic layer), striatum (external capsule), thalamic nuclei (paraventricular nuclei) and periventricular white matter.

Apoptosis was assessed by immunostaining with activated caspase-3 (Cas-3; 1:1000; R&D systems, Minneapolis, MN, USA) with cell counting performed on immunopositive cells. Oxidative stress was assessed using a marker of lipid peroxidation (4-hydroxynonenal; 4HNE; 1:500; Millipore, Billerica, MA, USA). Neuroinflammation was assessed using ionised calcium-binding adapter molecule 1 (IBA-1; Wako Pure Chemical Industries, Osaka, Japan) for the identification of activated microglia and macrophages. Sections underwent blockade of endogenous peroxidase activity by heating in citric acid buffer for 3×5 minutes, followed by an incubation in citric acid buffer (Cas-3: 30 minutes; 4HNE: 15 minutes; IBA-1: 20 minutes) and subsequent incubation in 3% hydrogen peroxide in 50% methanol (Cas-3: 30 minutes; 4HNE: 15 minutes; IBA-1: 30 minutes). Nonspecific binding was blocked by animal serum (Cas-3 & 4HNE: 5% normal goat serum and 1% bovine serum albumin; IBA-1: 10% normal goat serum) in 0.3% Triton X-100 phosphate buffer solution for 60 minutes. Slides were then incubated in primary antibody solution (Cas-3: 1:1000 in 5% normal goat serum, R&D Systems; 4HNE: 1:500 in antibody diluent, Dako, North Sydney, NSW, Australia; IBA-1 1:500, Wako Pure Chemical Industries). Caspase-3 and IBA-1 slides were incubated overnight at 4°C. 4HNE slides were incubated at room temperature for 1 hour then overnight at 4°C. Sections were then incubated in a 1:200 dilution of secondary antibody (Cas-3, 4HNE, and IBA-1: biotinylated goat anti-rabbit IgG antibody [Vector Laboratories, Burlingame, CA, USA]) in phosphate-buffered solution for 1 hour. Staining was visualised using 3,3′-diaminobenzidine (Pierce Biotechnology, Rockford, IL, USA). Immunopositive cells were counted under light microscopy (Olympus, Tokyo, Japan).

2.8 | Statistics

Data are presented as mean ± standard error of the mean. Blood gas parameters and plasma concentration of MDA, cytokines and MLT were analysed using 2-way (time and group) repeated measures ANOVA. One-way ANOVA with Tukey's multiple comparison post hoc analysis was used for cell counts, neurobehavioural assessments and MRS brain metabolites. Counts that failed a normality test were analysed using Kruskal-Wallis one-way ANOVA on ranks with Dunn's post hoc method. Significance was considered at $P < .05$.

3 | RESULTS

3.1 | Asphyxia and resuscitation

A total of 51 near-term lambs were used in the study, in which 49 survived the experimental period to 72 hours of life, Table 1. Two asphyxia-alone lambs died during the experimental period at 41 and 58 hours of life. These animals are not included in any analyses. There was no difference in birthweight between groups ($P = .16$), Table 1. All animals

TABLE 1 Asphyxia and resuscitation

	Control	Control+MLT	Asphyxia	Asphyxia+MLT	Asphyxia+MLT-P
Number	9	6	16	14	4
Male:female	5:4	1:5	6:10	8:6	3:1
In utero					
BP (mm Hg)	52 ± 3	50 ± 1	51 ± 2	45 ± 1	44 ± 2
HR (bpm)	207 ± 10	206 ± 15	193 ± 10	208 ± 14	213 ± 15
Asphyxia insult					
Asphyxia time (min)	–	–	9.9 ± 0.5	9.7 ± 0.3	9.1 ± 0.5
BP nadir (mm Hg)	–	–	12 ± 1	13 ± 1	14 ± 2
HR nadir (bpm)	–	–	94 ± 10	92 ± 8	86 ± 9
Resuscitation and ventilation					
Adrenaline required	0% (0/9)	0% (0/6)	25% (4/16)	29% (4/14)	25% (1/4)
Ventilation time (min)	21 ± 3	72 ± 34	**694 ± 380***	**78 ± 18#**	**93 ± 32#**
Weight					
Birthweight (kg)	4.2 ± 0.3	3.8 ± 0.5	3.5 ± 0.2	3.8 ± 0.2	3.9 ± 0.3
Post-mortem weight (kg)	4.5 ± 0.3	4.2 ± 0.5	**3.7 ± 0.2***	**4.5 ± 0.1#**	**4.3 ± 0.3#**

Characteristics of the asphyxia did not differ between groups. Asphyxia animals were significantly slower to attempt spontaneous respiration. All other resuscitation and ventilation parameters were not different between groups. Birthweight did not differ between groups, but by 72 h, asphyxia animals weighed significantly less than other groups having gained less weight since birth. Data presented as mean ± standard error of the mean. Significantly different values in bold. $*P < .05$ vs control. $\#P < .05$ vs asphyxia. MLT, Melatonin; BP, Blood pressure; HR, Heart rate.
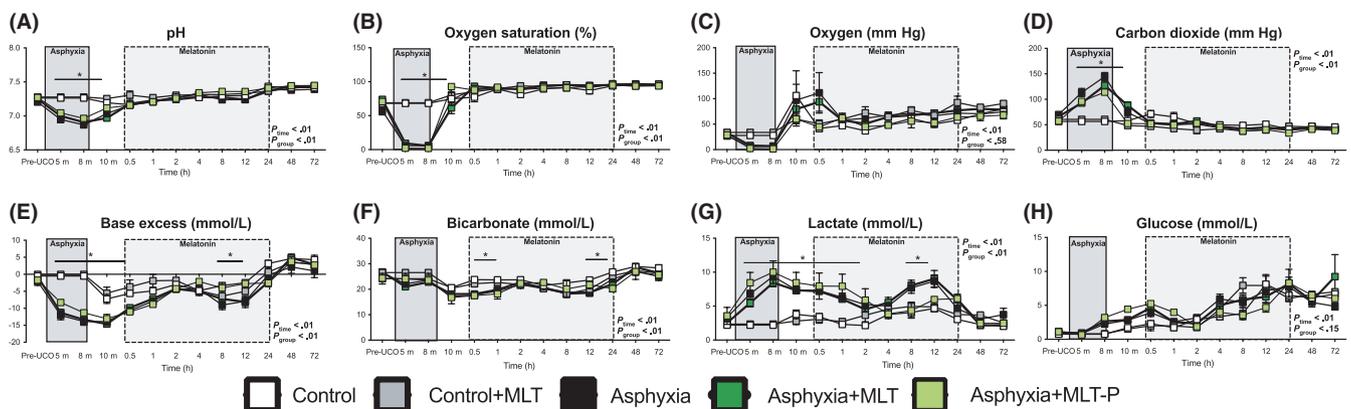

FIGURE 1 Physiological blood parameters over 72-h experimental period. (A) pH, (B) oxygen saturation (%), (C) partial pressure of oxygen (mm Hg), (D) partial pressure of carbon dioxide (mm Hg), (E) base excess (mmol/L), (F) bicarbonate (mmol/L), (G) lactate (mmol/L), (H) glucose (mmol/L). In utero, there was no difference between groups. Asphyxia led to a mixed respiratory-metabolic acidosis that resolved within 30 min of birth. Except lactate, that continued to be increased until 2 h postdelivery. A second derangement was seen between 8 and 24 h in base excess, bicarbonate and lactate. Data presented as mean ± standard error of the mean. $*P < .05$ is considered significant vs control

gained weight during the experimental period ($P_{time} < .01$) but, by 72 hours, asphyxia lambs weighed significantly less than other groups. Brain weights were not different between groups ($P = .31$).

In utero blood physiology parameters did not differ between animals. The duration of asphyxia was not significantly different between groups, Table 1. Asphyxia resulted in significant bradycardia and hypotension compared to in utero and those in control animals ($P < .01$), Table 1. In response to asphyxia, we observed a mixed respiratory-metabolic acidosis that was significantly deranged compared with baseline data and that in control animals, Figure 1. There was no significant difference in any parameter between asphyxia groups.

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3.2 | Melatonin

Melatonin concentrations in utero (prior to MLT administration) did not differ between groups (control: 0.04 ± 0.01 ng/mL; control+MLT: 0.08 ± 0.04 ng/mL; asphyxia: 0.20 ± 0.06 ng/mL; asphyxia+MLT: 0.24 ± 0.10 ng/mL; $P = .18$), Figure 2A. At 4 hours postbirth, and for the remainder of the time points examined, circulating MLT concentrations in control+MLT and asphyxia+MLT (IV) lambs were significantly higher than control-only animals by more than 600-fold. In asphyxia lambs alone, MLT concentrations were also significantly elevated by 3-fold at 4 hours and peaked with a 10-fold increase at 24 hours after birth, compared to control-only animals, Figure 2A. We confirmed that MLT concentrations were elevated in brain at 72 hours, as seen in Figure 2B, with MLT levels increased approximately 7-fold in control+MLT and asphyxia+MLT animals, Figure 2B.

The administration of MLT via a transdermal patch produced a 54-fold increase in circulating concentration at 4 hours, compared with control (27 ± 2 ng/mL vs 0.5 ± 0.3 ng/mL; $P = .02$), Figure 2A. By 12 hours postbirth, circulating MLT concentrations were not significantly different in control vs asphyxia+MLT-P lambs. New patches were administered at 12.5 hours, which resulted in a further rise in plasma concentration at 24 hours, a significant increase compared to control alone (26 ± 15 ng/mL vs 0.3 ± 0.1 ng/mL; $P = .01$). Within brain cortex, MLT concentrations were significantly increased in asphyxia+MLT-P animals compared with groups not treated with MLT (control: 0.08 ± 0.01 ng/mL; asphyxia+MLT-P 0.23 ± 0.05 ng/mL; $P < .001$), Figure 2B. The cortical concentrations of MLT were not significantly different between asphyxia+MLT and

asphyxia+MLT-P lambs (asphyxia+MLT: 0.46 ± 0.16 ng/mL; $P = .39$).

3.3 | Neurobehavioural milestones

Assessment of encephalopathy and neurodevelopmental wellbeing was scored over the experimental period in all lambs, Table 2. All control ± MLT lambs established formula bottle feeding, at a mean time of 2.1 ± 0.8 hours after birth. Control ± MLT lambs were all able to stand between 2 and 4 hours of birth, Table 2, and no seizures were observed in any control ± MLT lambs. The only difference noted between the control and the control+MLT lambs was that MLT-treated lambs spent a greater proportion of the first 24 hours sleeping (63% of the time, compared to 37% in control lambs; $P < .01$).

Asphyxia was associated with significant symptoms of encephalopathy, as shown in Table 2. Most asphyxia lambs (12 of 16) displayed abnormal tone (either hyper- or hypotonic) and poor reflexes at 12 hours after birth and had not established formula bottle feeding at 12 hours. The mean time to establish bottle feeding for asphyxia lambs, in those that could feed, was 20.5 ± 7 hours; $P = .04$ vs control. At 72 hours, 69% of asphyxia lambs could stand, with the mean time to achieve standing significantly delayed compared to control lambs (36.0 ± 6.2 hours vs 4.1 ± 0.9 hours; $P < .01$). Clinical seizures were observed in 38% (6 of 16) of lambs in the asphyxia group, and in these lambs, seizures were controlled with phenobarbitone.

Melatonin treatment was associated with improved neurological outcomes. Asphyxia+MLT lambs were more likely to demonstrate normal tone and a normal suckle reflex at
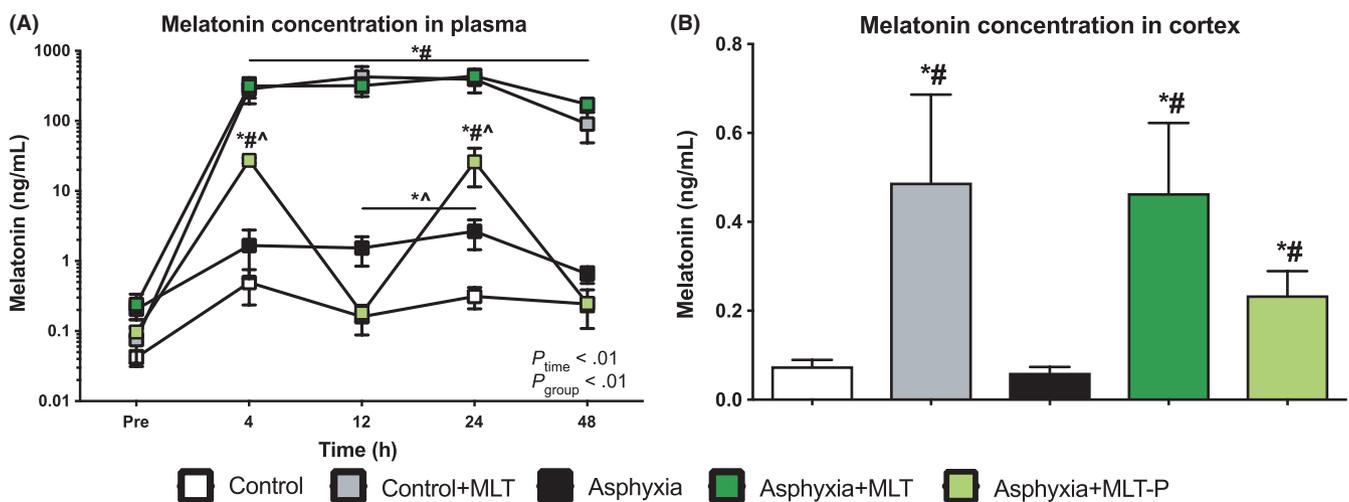

FIGURE 2 MLT concentration. (A) MLT concentration in plasma significantly increased by 4 h postdelivery in IV treated animals and remained elevated greater for more than 48 h. Transdermal administration resulted in significantly increased levels at 4 and 24 h. In asphyxia-alone animals, MLT was significantly increased at 12 and 24 h. (B) MLT was increased in the cortex of animals treated at time of post-mortem. Data presented as mean ± standard error of the mean. *$P < .01$ vs control. #$P < .01$ vs asphyxia. ^$P < .01$ vs asphyxia+MLT

TABLE 2 Neurobehavioural milestones

	Control	Control+MLT	Asphyxia	Asphyxia+MLT	Asphyxia+MLT-P
Feeding					
Glucose infusion required	33% (3/9)	0% (0/6)	**100% (16/16)***	**71% (10/14)#**	**100% (4/4)#**
Established formula feeding	100% (9/9)	100% (6/6)	80% (13/16)	100% (14/14)	100% (4/4)
Time of establishing feeding (h)	2.1 ± 0.8	2.1 ± 0.6	**20.5 ± 7.0***	6.4 ± 1.5#	7.6 ± 2.0#
Normal suckle					
At 12 h	100% (9/9)	100% (6/6)	**38% (6/16)***	**79% (11/14)#**	**75% (3/4)#**
At 72 h	100% (9/9)	100% (6/6)	63% (10/16)	86% (12/14)	75% (3/4)
Normal tone					
At 12 h	100% (9/9)	100% (6/6)	**38% (6/16)***	**79% (11/14)#**	**75% (3/4)#**
At 72 h	100% (9/9)	100% (6/6)	50% (8/16)	86% (12/14)	100% (4/4)
Standing					
First attempted (h)	2.3 ± 0.5	4.3 ± 1.4	**27.9 ± 6.9***	10.8 ± 4.8	11.3 ± 1.5
Attained (h)	4.1 ± 0.9	6.5 ± 1.9	**36 ± 6.2***	20.0 ± 5.5	23.0 ± 5.0
Ability at 12 h	100% (9/9)	100% (6/6)	**38% (6/16)***	79% (11/14)	75% (3/4)
Ability at 72 h	100% (9/9)	100% (6/6)	69% (11/16)	93% (13/14)	100% (4/4)
Seizures					
Present over experiment	0% (0/9)	0% (0/6)	**38% (6/16)***	7% (1/14)	0% (0/4)
Presence by 12 h	0% (0/9)	0% (0/6)	**38% (6/16)***	7% (1/14)	0% (0/4)
Presence by 72 h	0% (0/9)	0% (0/6)	31% (5/16)	7% (1/14)	0% (0/4)
Sleep:Wake cycle over 24 h					
% of time asleep	37	**63***	**66***	**84*#**	**74*#**

Asphyxia was associated with symptoms of encephalopathy related to deficits in feeding, tone/posture and clinical seizures. MLT (MLT) treatment significantly improved these characteristics. Data presented as mean ± standard error of the mean. Significantly different values in bold. $P < 0.05$ is considered significant. *$P < .01$ vs control. #$P < .01$ vs asphyxia.

12 hours (11 of 14 lambs), compared to asphyxia lambs ($P < .05$). The mean time to establish formula feeding in asphyxia+MLT lambs was 6.4 ± 1.5 hours; $P = .04$ vs asphyxia. At 12 hours, 79% of asphyxia+MLT lambs could stand, double the number of animals in the asphyxia-alone group (38%; $P < .05$). MLT treatment significantly decreased the likelihood of seizures; 1 of 14 lambs in the asphyxia+MLT group demonstrated clinical seizure activity. Finally, asphyxia+MLT lambs spent a significantly more time asleep in the first 24 hours after birth (84%) compared to untreated asphyxia and control lambs (66% and 37%, respectively; $P < .05$).

Asphyxia+MLT-P lambs showed a similar degree of improvement in neurobehavioural milestones as did asphyxia+MLT lambs, Table 2. All asphyxia+MLT-P lambs (4 of 4) established feeding by 72 hours. Seventy-five per cent of asphyxia+MLT lambs demonstrated a normal suckle reflex at 12 and 72 hours and normal tone at 12 hours, while all animals had normal tone and were standing at 72 hours. The mean time to achieve standing was 23 ± 5 hours; $P = .3$ vs

asphyxia. No asphyxia+MLT-P lambs had clinically overt seizures.

3.4 | Magnetic resonance spectroscopy

Brain metabolite markers of hypoxia and injury were detected via MRS at 12 and 72 hours after birth, Figure 3. At 12 hours, the lactate:NAA ratio was increased 2.5-fold in asphyxia lambs compared to controls (0.20 ± 0.06 vs 0.08 ± 0.02; $P = .04$) an increase not seen with MLT (asphyxia+MLT 0.02 ± 0.06). We also observed a significant difference in the lactate:NAA ratio between asphyxia and asphyxia+MLT lambs at 12 hours ($P = .04$), Figure 3C. At 12 hours, neither the measures of anaerobic metabolism (lactate:choline ratio) nor measures of healthy neurons (NAA:choline ratio) were significantly different between groups, Figure 3A,D. At 72 hours, the ratio of lactate:NAA was increased over 3-fold in asphyxia lambs compared to controls (0.37 ± 0.16 vs 0.10 ± 0.04; $P < .01$ an increase not seen with MLT (asphyxia+MLT 0.10 ± 0.04; $P = .02$),
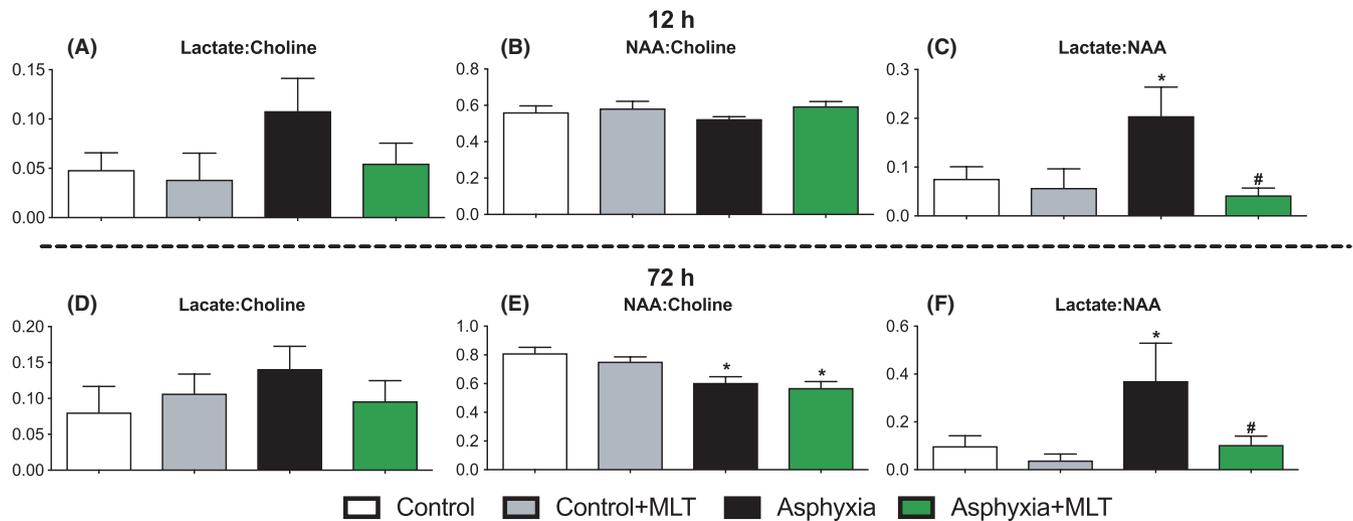
FIGURE 3 Magnetic resonance spectroscopy parameters. 12 h: (A) lactate:choline (B) N-acetylaspartic acid (NAA):choline (C) lactate:NAA. 72 h (D) lactate:choline (E) NAA:choline (F) lactate:NAA. Asphyxia led to significant derangement of MRS parameters at 12 h, lactate: NAA and at 72 h, NAA:choline, lactate: NAA. Data presented as mean ± standard error of the mean. $P < .05$ is considered significant. $*P < .01$ vs control. $\#P < .01$ vs asphyxia

Figure 3F. At 72 hours, the ratio of NAA:choline was also significantly reduced in all asphyxia animals (asphyxia: 0.60 ± 0.04; asphyxia+MLT: 0.57 ± 0.05) significantly reduced compared with control groups (control: 0.81 ± 0.04; control+MLT: 0.75 ± 0.03; $P < .01$), Figure 3E.

3.5 | Biochemical neuropathology markers in cerebrospinal fluid

Cerebrospinal fluid was collected at 72 hours to measure markers of oxidative stress (MDA), inflammatory cytokines (IL-1B, IL-10, IL-6 and TNF-A) and neuropathology/astrogliosis (S100B). MDA was significantly increased in asphyxia animals (14.0 ± 3.4 nmol/mL) compared with control and control+MLT (control: 5.9 ± 1.0 nmol/mL; control+MLT: 5.7 ± 1.0 nmol/mL; $P < .01$). IV MLT treatment significantly reduced MDA concentrations in CSF (asphyxia+MLT 6.6 ± 1.7; $P < .01$ vs asphyxia). CSF IL-1B was significantly increased in asphyxia animals (86 ± 38 pg/mL) compared with control, control+MLT and asphyxia+MLT (all readings below sensitivity of the assay of 0.274 pg/mL). Other inflammatory cytokines read below the assay sensitivity within CSF at 72 hours, in all groups. S100B was significantly increased in CSF collected from asphyxia animals (193 ± 40 μg/mL; $P < .01$ vs control 29 ± 12 μg/mL and control+MLT 40 ± 20 μg/mL). CSF S100B was reduced in asphyxia+MLT lambs (61 ± 21 μg/mL; $P = .003$ vs asphyxia) and was not different from control lambs ($P = .23$).

The administration of MLT via transdermal patch reduced CSF lipid peroxidation as measured via MDA concentration. MDA was significantly decreased in asphyxia+MLT-P lambs compared to asphyxia (7.2 ± 2.5 vs 14.0 ± 1.3, respectively; $P = .02$), and asphyxia+MLT-P was not significantly different to controls (control: 5.9 ± 1.0 nmol/mL; $P = .3$). Cytokines were not detectable in the CSF of asphyxia+MLT-P lambs at 72 hours. The concentration of S100B in CSF of asphyxia+MLT-P lambs was not different to controls ($P = .12$) and was significantly decreased compared with asphyxia animals (asphyxia: 193 ± 40 μg/mL vs asphyxia+MLT-P: 82 ± 39; $P = .02$).

3.6 | Neurohistopathology

Asphyxia was associated with widespread grey and white matter cellular apoptosis, oxidative stress and neuroinflammation, Figure 4. Activated caspase-3-mediated apoptosis was significantly increased across all grey and white matter brain regions examined in response to asphyxia, when compared with basal levels in control brains, Figure 4A-E,P. We observed a significant reduction in cell counts for caspase-3-mediated apoptosis in asphyxia+MLT brains compared to asphyxia lambs, across all regions examined, and cell counts for caspase-3 were not different in asphyxia+MLT lambs compared with control ± MLT lambs, for all brain regions analysed. MLT patch administration demonstrated an intermediate improvement in apoptosis-mediated cell death. Compared to asphyxia, asphyxia+MLT-P reduced caspase-3 cell counts within the hippocampus, thalamus, white matter and the striatum but not the cortex. Apoptotic cell counts were not significantly different between asphyxia+MLT-P and control brains for all regions examined.

Cerebral oxidative stress was assessed via 4HNE immunohistochemistry for cellular lipid peroxidation, Figure 4F-J,Q. Immunostaining for 4HNE was significantly increased
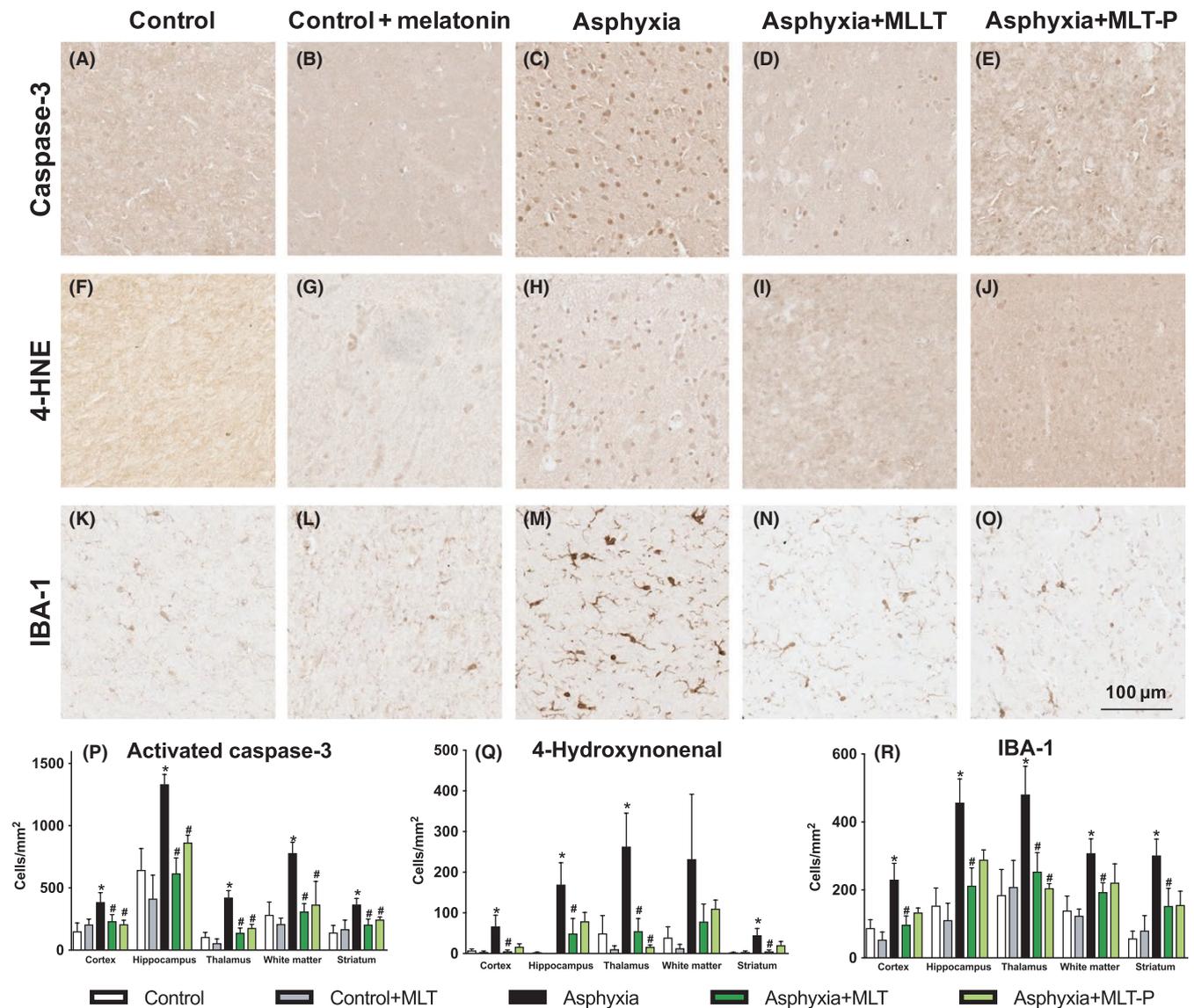
FIGURE 4 Histological cell counts of apoptosis (caspase-3; A), lipid peroxidation (4-hydroxynonenal; B), and macrophages and microglia (IBA-1; C) measured in the midtemporal cortex, dentate gyrus of the hippocampus, paraventricular thalamic nuclei, periventricular white matter and striatum. *P < .01 vs control. #P < .01 vs asphyxia

in the hippocampus, cortex and grey matter in response to asphyxia compared with control ± MLT animals. We observed a significant reduction in cell counts for oxidative stress in the asphyxia+MLT brains across most regions examined compared with asphyxia-alone animals. Cell counts for 4HNE were not significantly different between asphyxia+MLT animals compared with control ± MLT animals. MLT patch administration normalised 4HNE cell counts within the thalamus, compared with asphyxia alone, and in no region were asphyxia+MLT-P cell counts significantly different to control ± MLT.

Inflammatory cells were assessed by cell counts of macrophages and activated microglia infiltration, using IBA-1 immunohistochemistry, Figure 4K-O,R. A significantly higher number of infiltrating macrophages and microglia were

observed in asphyxia brains compared with control ± MLT. Asphyxia+MLT inflammatory cell counts were not significantly different compared with control ± MLT animals but were significantly decreased compared with asphyxia-alone. Asphyxia+MLT-P animals had significantly reduced inflammation within the thalamus compared with asphyxia-alone animals. There was no increase in inflammation in Asphyxia+MLT-P animals in any region compared with control ± MLT.

4 | DISCUSSION

Perinatal asphyxia and subsequent HIE remain global burdens that contribute substantially to neonatal death or, in

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survivors, long-term disability. A simple, cheap and effective postnatal intervention could have a profound impact on neonatal survival and quality of life for many thousands of children. Here, we show that melatonin (MLT) is neuroprotective for perinatal asphyxia. In our lamb model of perinatal asphyxia, we commenced treatment 30 minutes following asphyxia at birth and administered MLT intravenously (IV) or transdermally over 24 hours. MLT significantly improved the attainment of functional developmental milestones postbirth and normalised cerebral markers of deranged brain metabolism to control levels, assessed using MRS. At the cellular level, MLT treatment resulted in a significant reduction in apoptosis-mediated cell death and reduced cerebral oxidative stress and inflammation following asphyxia. Results from this study confirm the ease of administration of MLT and strongly support a clinical study for MLT treatment of perinatal asphyxia in low- and middle-income birth settings. Notably, we show that a simple MLT skin patch has significant neuroprotective benefits.

Functional assessments of the lambs were undertaken over the experimental period to examine for signs of encephalopathy. Control and control+MLT lambs fed well from a bottle, all had normal tone and stood within 6 hours of birth in the absence of seizures. Asphyxia induced significant deficits, with 50% of lambs demonstrating abnormal tone (hyper- or hypotonic), 31% of lambs unable to stand at 72 hours and a significant delay to achieve a standing position in the remaining animals. MLT improved all neurodevelopmental assessments following asphyxia. Clinical seizures were present in 38% (6 of 16) asphyxia lambs, but only in 6% of melatonin-treated lambs (1 of 18, IV or patch). The presence of seizures within the first day of life is strongly linked to poor outcome and nearly half of infants with seizures will be severely developmentally delayed.[52] In addition, we assessed the time that lambs spent asleep over the duration of MLT treatment, that is, the first 24 hours. MLT induced sedative effects; control+MLT lambs slept 63% of the time over the 24 hours, compared with 37% in control animals. Asphyxia-alone lambs spent 66% of their time sleeping, and this was increased with MLT treatment (asphyxia+MLT: 84%; asphyxia+MLT-P: 74%). The functional/behavioural improvements observed with MLT treatment of newborn lambs provide a preclinical model which confirms and extends results from newborn rats exposed to hypoxia-ischaemia, in which impaired ability to acquire and retain spatial memory tasks was mitigated by MLT administration postinsult.[53] Our study is the first to demonstrate that MLT treatment improves functional outcomes in a large animal model of term perinatal asphyxia.

The term newborn brain is highly susceptible to hypoxic-ischaemic injury due to immature innate defence systems, contributed by immature excitatory receptors, limited anti-inflammatory and antioxidant capacity and a high metabolic demand for continuing brain development.[12-14,54] In the current study, we demonstrate that MLT inhibited oxidative stress within the term brain in response to acute severe asphyxia. Asphyxia increased levels of oxidative stress measured biochemically by MDA within the CSF at 72 hours (at post-mortem) and histologically via 4HNE upregulation within both white and grey matter regions of the brain. Aldehydes, such as 4HNE and MDA, are highly reactive and toxic and induce cell death and axonal injury within the brain.[7] Both IV and transdermal MLT treatment decreased aldehyde levels, an effect likely mediated by MLT's ability to directly scavenge ROS, upregulate antioxidant enzymes and stabilise mitochondrial function.[11] We have previously shown a biphasic temporal pattern of ROS production (hydroxyl radical) in cerebral grey matter following acute hypoxia-ischaemia; an early phase soon after insult, and a secondary phase at 6-8 hours, and both peaks were prevented with MLT treatment.[11] Clinically, asphyxia in term neonates induces a 2-fold increase in circulating MDA concentration, compared with healthy controls, which remains elevated for 12-24 hours in the CSF following an insult.[55] In response to acute hypoxic or inflammatory stimuli, the brain's resident immune microglial cells become activated and proliferate, as was observed in the current study. Activated microglia release cytotoxic compounds, which include ROS and pro-inflammatory cytokines,[56] in keeping with the elevation of IL-1B and the aldehyde MDA in CSF of asphyxia lambs seen here. This neuroinflammatory response is well described in animal models of perinatal asphyxia, commencing almost immediately after insult and manifesting over days to weeks[56] and acting as a principal mediator of neuronal injury.[57-59] Similarly, microglial activation and cytokine release are observed over the same timescale in human infants after perinatal asphyxia.[60,61] MLT administration (IV and transdermal patch) prevented the neuroinflammatory response following asphyxia, with undetectable levels of IL-1B in CSF and a total reduction in the number of activated microglia within brain regions of interest. These results add critical weight to evidence that the antioxidant and anti-inflammatory actions of MLT are central to its neuroprotective benefits, which in turn rescue functional development following birth asphyxia.

Our model of perinatal asphyxia in lambs produces a pattern of neuropathology involving significant apoptosis within the cortex, white matter and deep grey matter structures.[9,43] This pattern of brain injury is consistent with the selective neuronal cell loss observed within these brain regions in the human infant.[62-65] MLT prevented apoptosis within the cortical grey matter, deep grey matter and white matter of asphyxic lambs. S100B is also used as a neuropathology marker as it is released from damaged neurons or astrocytes[43,66] and is increased in newborns following perinatal asphyxia.[67,68] In CSF collected at 72 hours, we detected a significant elevation in S100B protein following asphyxia, which was ameliorated

with MLT treatment. Cellular protection may be mediated by MLT's ability to decrease and/or block protease inhibitors that play an essential role in programmed cell death[69-71] and necrotic cell death.[11,72,73] Our results strongly support that MLT promotes neuronal survival when commenced early after an asphyxic event.

Our preclinical lamb model of acute perinatal asphyxia causes a significant metabolic acidosis, pH < 6.9 and base excess less than −14 mmol/L, consistent with the pattern seen following severe asphyxia in the human and replicates the resuscitation and postnatal care required in the clinical situation.[74,75] Blood lactate, a product of anaerobic metabolism, was slow to recover to baseline and then demonstrated a secondary derangement at 8-12 hours following birth. Blood lactate is considered a good marker of hypoxic-ischaemic insult and prognosis, correlating well with the extent of subsequent neuronal degeneration.[43,76] However, the commencement of MLT at 30 minutes did not alter the blood lactate response. This suggests that the primary mechanism of MLT action is via its brain antioxidant and anti-inflammatory actions and not due to systemic metabolic improvement.

Clinically, MRS assessment of brain lactate:NAA ratio is a particularly useful brain metabolic biomarker in infants with HIE, with a 2-fold increase in lactate:NAA following perinatal asphyxia associated with poor neurodevelopmental outcome at 18 months of age.[77-79] We observed a 2.5- to 3.5-fold increase in brain lactate:NAA ratio at 12 and 72 hours, respectively, following perinatal asphyxia in lambs. That MLT restored cerebral lactate:NAA to control levels in asphyxia+MLT lambs suggests that it may be effective in improving long-term functional outcomes, not just the short-term outcomes that we were able to assess in this study. The normalisation of lactate:NAA by MLT was principally mediated by MLT modifying the cerebral lactate response (lactate:choline), as MLT did not alter the decrease in NAA:choline at 72 hours. This is an intriguing observation, as NAA is considered marker of neuronal integrity, wherein a reduction in NAA purportedly reflects neuronal damage.[79] However, NAA:choline is not a good prognostic indicator on early MRS in newborns with suspected perinatal asphyxia.[78,79] It is possible that reduced NAA in the brain of asphyxia ± MLT lambs at 72 hours after asphyxia indicates altered neuronal metabolism or function, rather than cell damage.[80]

This study was designed to address the neuroprotective capacity of MLT, administered soon after term perinatal asphyxia as a stand-alone therapeutic intervention that might be applicable in a low-resource setting, or where access to tertiary care is limited. We purposefully gave MLT in the absence of neonatal hypothermia so that we could assess MLT as a stand-alone intervention, with comparison of 2 routes of administration. While both routes of MLT administration, IV and transdermal patch, were neuroprotective, the histology results suggested a somewhat blunted effect of transdermal

delivery compared to IV administration. This is potentially due to more labile plasma MLT levels. Although a total of 60 mg of MLT over the first 24 hours after birth were administered in both treatments, for transdermal administration, we used commercially available MLT patches that raised circulating MLT concentration 55-fold greater than in asphyxia-alone animals at 4 hours. At 12 hours, circulating MLT levels had returned to baseline in patch lambs, but a new set of patches again significantly increased MLT concentrations at 24 hours. Patch administration increased cerebral cortex MLT concentration 3-fold compared to control animals. In contrast, intravenous MLT administration every 2 hours induced a sustained increase in circulating MLT concentrations >600-fold, and a further 2-fold increase in the cerebral cortex compared with patch administration. In adult studies, the maximum concentrations of MLT are reached with transdermal patches between 1 and 18 hours following application.[45-49,81] These human studies observed inconsistent concentrations of MLT between patches; generally, the higher dose patches showed a faster time to maximum concentration, for example 20-100 mg patches produced a peak circulation at 1-2 hours.[47] We undertook a small in vitro study to assess MLT release from the patches used in this study and found that MLT patches had a maximal diffusion rate of approximately 875 pg/mL/h (data not shown) reached at 2-4 hours, but this was highly variable. While outside the scope of this study, it is interesting to note the biochemistry of melatonin metabolism; melatonin is readily metabolised by the liver and skin into further potent antioxidant compounds such as 6-hydroxymelatonin and N1-acetyl-N2-formyl-5-methoxykynuramine,[21,22] which may mediate some of the observed neuroprotective effects. Indeed, local melatonin metabolism may partially account for a lower circulating melatonin concentration following patch delivery, but with a similar level of neuroprotective benefit due to antioxidant activity of metabolites.

Importantly, transdermal administration of MLT has many benefits that support its use in low- and high-resource countries as an intervention for perinatal asphyxia. MLT patches are easy to apply, painless, temperature stable and have potential for sustained slow release, and patch delivery maintains the integrity of the skin to minimise infection risk.[82] Furthermore, an ongoing concern with IV administration of MLT is that ethanol is the commonly used excipient,[83] which is unsuitable for administration to newborn infants. The development of a MLT IV formulation or reliable slow-release patch that does not contain ethanol should be a priority for use in newborn infants.

The term newborn infant does not ordinarily produce significant levels of MLT for weeks to months after birth.[16,84] This may reduce the newborn's endogenous antioxidant capacity. In the newborn lambs in the current study, we did, however, note that asphyxia alone induced a 10-fold increase

in plasma MLT concentration. This accords with previous observations that endogenous levels of MLT are increased following a severe hypoxic event.[16,73] This endogenous MLT rise is not sufficient to confer neuroprotective benefits, as evidenced by significant cell death in the brain of asphyxia-alone lambs. We commenced MLT treatment at 30 minutes postdelivery, which is earlier than in some other studies to date.[3,85] Although translation of early MLT patch therapy into the clinic will require adequate training of staff in the early diagnosis of at-risk infants with signs of encephalopathy,[86] this is a reasonable timeframe in which an "at-risk" newborn infant could have a patch administered and early administration ensures that the initial ROS peak within the brain, observed within 30 minutes after insult,[11] is mitigated.

There is now a strong body of experimental evidence to support MLT as a neuroprotective treatment for preterm and term hypoxic-ischaemic brain injury, whether administered as a prophylactic or postinsult treatment, principally mediated via MLT's antioxidant, antiapoptotic and anti-inflammatory properties.[16,30,50,73,87,88] This study aimed to determine the effectiveness of MLT treatment for term asphyxia in a preclinical lamb model, which could be applied in a low-resource birth setting. Our results strongly support that MLT administration is neuroprotective as a stand-alone treatment for perinatal asphyxia, mediated via cerebral antioxidant and anti-inflammatory actions. This is the first study to show that neonatal application of MLT has excellent stand-alone neuroprotective benefits and improves functional outcome in a preclinical model of perinatal asphyxia. This has broad application potential for low-resource countries where the rates of perinatal asphyxia remain very high, and the administration of melatonin as a transdermal patch is feasible and likely to be efficacious. Developing a stable, controlled release melatonin patch should now be a priority research area.

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