MELATONIN AUGMENTS THE NEUROPROTECTIVE EFFECTS OF HYPOTHERMIA IN LAMBS FOLLOWING PERINATAL ASPHYXIA

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/JPI.12744

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**Conflict of interest statement:** The authors have declared that no conflict of interest exists.

**Keywords:** Perinatal asphyxia, therapeutic hypothermia, neonatal encephalopathy, neuroprotection, melatonin, oxidative stress, brain injury.

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

**ABSTRACT**

Therapeutic hypothermia (TH) is standard care in high resource birth settings for infants with neonatal encephalopathy. TH is partially effective and adjuvant therapies are needed. Here we examined whether the antioxidant melatonin (MLT) provides additive benefit with TH, compared to TH alone or MLT alone, to improve recovery from acute encephalopathy in newborn lambs. Immediately before caesarean section delivery, we induced asphyxia in fetal sheep via umbilical cord occlusion until mean arterial blood pressure fell from 55 ± 3 mmHg in sham controls to 18-20 mmHg (10.1±1.5 min). Lambs were delivered and randomized to control, control+MLT (60mg i.v., from 30 min to 24 h), asphyxia, asphyxia+TH (whole-body cooling to 35.1±0.8°C vs 38.3±0.17°C in sham controls, from 4-28 h), asphyxia+MLT, and asphyxia+TH+MLT. At 72 h, magnetic resonance spectroscopy (MRS) was undertaken and then brains collected for neuropathology assessment. Asphyxia induced abnormal brain metabolism on MRS with increased Lactate:NAA (p=0.003) and reduced NAA:Choline (p=0.005), induced apoptotic and necrotic cell death across grey and white matter brain regions (p<0.05), and increased neuroinflammation and oxidative stress (p<0.05). TH and MLT were independently associated with region-specific reductions in oxidative stress, inflammation, and cell death, compared to asphyxia alone. There was an interaction between TH and MLT such that the NAA:Choline ratio was not significantly different after asphyxia+TH+MLT compared to sham controls, but had a greater overall reduction in neuropathology than either treatment alone. This study demonstrates that, in newborn lambs, combined TH+MLT for neonatal encephalopathy provides significantly greater neuroprotection than either alone. These results will guide development of further trials for neonatal encephalopathy.

**INTRODUCTION**

Neonatal encephalopathy describes acute, evolving abnormal neurological function in the days after birth. The etiology of neonatal encephalopathy is most commonly severe acute hypoxic-ischemic or...
asphyxic episode around the time of birth but may also involve other factors and hence the move away from the older term, hypoxic-ischaemic encephalopathy (HIE)\(^1\). In all birth settings, neonatal encephalopathy is a primary cause for premature death or long-term disability. Therapeutic hypothermia (TH) is standard care for term infants with moderate to severe neonatal encephalopathy in high income countries\(^2\). Reducing infant body temperature by 3.5 ± 0.5 °C (i.e. to 33.5–34.5°C), commencing within 6 hours of birth and continuing for 72 hours, reduces mortality and moderate-to-severe neurodevelopmental disability at 18 months of age \(^2\)\(^-\)\(^4\). Nevertheless, benefits of TH for infants with neonatal encephalopathy are only partial, and in the original randomized controlled trials, nearly half of encephalopathic newborns treated with TH died, or survived with disabilities\(^4\). Improved outcomes for infants with neonatal encephalopathy require adjuvant approaches to augment hypothermic neuroprotection.

Melatonin (MLT) is a candidate neuroprotective treatment due to its potent antioxidant properties. MLT is safe in pregnancy and in the neonate and readily crosses the blood-brain barrier\(^5\)\(^-\)\(^9\). In preclinical fetal sheep studies, MLT attenuates brain hydroxyl radical production following hypoxia-ischemia and reduces markers of circulating oxidative stress, improves functional recovery of brain activity, and reduces brain inflammation and cell death\(^5,6,10\). Following asphyxia at birth in lambs, MLT is an effective stand-alone neuroprotective therapy, preventing brain metabolic dysfunction on magnetic resonance spectroscopy (MRS) and reducing neuropathology\(^11\). In a piglet model of neonatal encephalopathy after hypoxia-ischemia, the Robertson group were the first to demonstrate that MLT combined with TH significantly improved MRS-indices of brain metabolism and reduced cell death\(^12,13\). There are no studies to date in which TH alone, MLT alone, and TH+MLT have been assessed head-to-head, to directly compare the separate and additive effects of these therapies on neuropathology and functional outcomes subsequent to perinatal asphyxia.

Our aim in the current study was to examine in lambs whether MLT enhanced the neuroprotective benefits of TH following asphyxia at birth, compared to either TH alone or MLT alone. We tested the hypothesis that MLT would provide additive neuroprotective benefit to TH following asphyxia at birth, and that TH+MLT together would provide greater neuroprotection than either treatment alone, using our well characterized near-term lamb model of asphyxia at birth\(^11,14,15\). This study included a total of 6 cohorts of lambs: control, control+MLT, asphyxia, asphyxia+MLT, asphyxia+TH, asphyxia+TH+MLT, in order to assess the separate effects of TH and MLT, plus their combined actions.
MATERIALS & METHODS

Experiments were undertaken following the Australian NHMRC code for the care and use of animals for scientific purposes and had institutional approval. Animals were sourced from the Monash Animal Research Platform.

Surgery

Pregnant ewes at 139–141 days gestation (term is ~145d) under general anesthesia underwent surgery induced by sodium-thiopentone (20mg/kg IV; Pentothal, Boehringer Ingelheim, Australia) and maintained with 1–2.5\% isoflurane (IsoFlo, Abott Laboratories, USA) in oxygen/nitrous oxide (O\(_2\): 2–L; N\(_2\)O: 1L). With the lower limbs of the lamb exposed from the uterus, femoral artery and vein catheters were inserted. Arterial blood samples were collected before asphyxia, at 8min during occlusion, then at 4, 12, 24, 48 and 72h post asphyxia and delivery.

Asphyxia and resuscitation

Asphyxia was induced in fetal lambs by complete cord occlusion\textsuperscript{1,14,15} until arterial blood pressure fell to 18-20\,mmHg, compared to 55 ± 3\,mmHg in sham controls. Lambs were delivered and resuscitated with endotracheal intubation and positive pressure ventilation (NeoPuff, Fisher and Paykel Healthcare, Australia; 30\,cm H\(_2\)O PIP; 5-8\,cm PEEP; 10\,L/min room air; 30\,breaths/min), and then ventilated (Babylog 8000+, Dräger, Australia; volume guarantee 5\,mL/kg) until the lamb commenced spontaneous breathing >50\% of the time, after which ventilation was ceased.

Neuroprotection strategies

Hypothermia

Whole body TH to 35±0.5\,°C was undertaken for 24h on asphyxia, asphyxia+TH and asphyxia+TH+MLT lambs. This represented a target reduction of 3.5\,°C below normal temperature of ~38.5\,°C in the lamb. At 3.5\,h after birth, the lamb’s belly was shaved, and a rectal temperature probe (ADI Instruments, Australia) was placed. At 4h after birth, TH was induced using crushed ice within large zip lock bags around the conscious lamb. Following 24\,h of TH (Table 1), the lamb was rewarmed 0.5\,°C/h over a 10\,h period to ~39\,°C.

Melatonin

MLT (Sigma-Aldrich, Australia) was administered IV to control+MLT, asphyxia+MLT and asphyxia+TH+MLT lambs. MLT was prepared in absolute ethanol (5\% final volume) and saline as described previously\textsuperscript{11} and administered via the femoral vein in 5\,mg boluses, starting 30\,min post birth, and then every 2\,h until 24.5\,h, for a total dose of 60\,mg over 24\,h. We have previously shown this
dosing regimen to provide a sustained increase in circulating and brain melatonin that represents a neuroprotective dose\textsuperscript{11}.  

**Maintenance**

Blood sampling was used for clinical management (e.g. glucose and bicarbonate) and sheep milk formula (Wombaroo, Glen Osmond, South Australia, Australia) was provided once lambs had attained good suckling reflex. Until that time lambs were given maintenance fluids (10% glucose, 40ml/kg/d).

**Behavioral milestones**

Signs of encephalopathy were characterized using a modified Sarnat system\textsuperscript{14-16}, including ability to stand and feed, and seizure-like activity. Evidence of clinical seizures (jerking, spasms, stiffening of limbs)\textsuperscript{17} required treatment with 20mg/kg phenobarbitone, administered IV over 30 min. Percentage of time spent asleep over the first 24h was noted.

**Magnetic resonance spectroscopy**

At 72h, lambs were lightly sedated (medetomidine hydrochloride 0.1mg/kg, Domitor, Pfizer, Australia) and MRI/MRS undertaken using a 3T Siemens Vario (Siemens Medical Solutions, PA, USA) with an 8-channel knee coil (400x420x310 mm). MRS was performed using a 270 ms echo time, 2cm\textsuperscript{3} voxel placed over deep grey matter containing hippocampus, striatum and basal ganglia for assessment of choline (cho), n-acetyl aspartate (NAA), and lactate (lac) with computer generated area under the curve analysis, and ratios were determined.

**Biological fluid markers of neuropathology**

MLT concentrations in plasma (fetal levels pre asphyxia, and 4h, 12h, 24h and 48h) and cerebral cortex of the brain were assessed by radioimmunoassay kit as per manufacturer’s instructions (Buhlmann Laboratories, Schonenbuch, Switzerland) with a sensitivity of 0.3pg/ml, intra-assay precision of 7.9% and inter-assay precision of 11.7%. Cerebrospinal fluid (CSF) was collected at post mortem and concentrations of malondialdehyde (MDA) measured using a thiobarbituric acid reactive substances (TBARS) assay\textsuperscript{9} ( assay sensitivity of 0.1µmol/L, intra-assay precision 3.1% and inter-assay precision 5.1%), S100 calcium binding protein B (S100B), (DiaSorin, Stillwater, USA; assay sensitivity 0.03µg/L, intra-assay precision <10%, inter-assay precision <15%) and cytokines (IL-1\textbeta, IL-6, IL-10, and TNF-\textalpha) were also measured (Cytokine Bead Array, BD Biosciences, North Ryde, Australia; >85% recovery of standards, sensitivity of 0.274pg/ml, intra-assay precision for IL-1\textbeta 157%, IL-6 164%, IL-10 120%, TNF-\textalpha 116%).

**Neurohistopathology**

At 72h, after the MRS measurements, lambs were killed (5mL pentobarbitone; 400mg/kg, Lethabarb, Virac, Australia), and brains removed and weighed. The right brain hemisphere was cut into 5mm

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slices coronally and fixed in 4% paraformaldehyde for 48h, followed by paraffin embedding and coronal section at 10μm. Brain regions were analyzed at the perirolandic cortex, including hippocampus (CA1), cortex (molecular layer), thalamus (paraventricular nuclei), striatum (external capsule), and subcortical white matter. Immunohistochemistry was carried out as previously described\textsuperscript{11,14,15} with lipid peroxidation analyzed using 4-hydroxynonenal (4-HNE; 1:500; Millipore, USA), all IBA-1-positive microglia and macrophages assessed using ionized calcium-binding adapter molecule (IBA-1; 1:500; Wako Pure, Osaka, Japan), apoptotic cell death assessed using activated caspase-3 (Cas-3; 1:1000, R&D Systems, MN, USA), and necrosis assessed via cresyl violet-acid fuchsin (Amber Scientific, Midvale WA, Australia) staining to identify organelle swelling, pyknotic nuclei, eosinophilic cytoplasm, or cells with condensed cytoplasm\textsuperscript{18,19}. The average number or percent area coverage of positively stained cells across three fields of view were calculated using ImageJ (v1.48, NIH).

**Statistical analyses**

Data are mean ± SEM, with significance accepted when p<0.05. Analysis was undertaken using GraphPad Prism 8 (GraphPad Software, USA). A two-step analysis was performed, firstly examining asphyxia versus control (control, asphyxia, control+MLT) via 1-way ANOVA, followed by 2-way ANOVA with TH and MLT as independent variables to assess outcomes within the asphyxia groups (asphyxia, asphyxia+TH, asphyxia+MLT, asphyxia+TH+MLT). Repeated measures analysis was included for continuous data. Where a significant effect of the independent variables was found, post hoc comparisons were made using Tukey’s multiple comparisons for normally distributed data. Non-parametric data were analyzed using Kruskal-Wallis ANOVA on ranks, with Dunn’s method.

**RESULTS**

**Asphyxia and resuscitation**

Baseline group characteristics are shown in Table 1. Blood gas and physiological parameters were not different between groups before asphyxia. Asphyxia induced severe metabolic acidosis in all groups consistent with the clinical criteria for a severe acute hypoxic event at birth, pH<7 and base excess <-12\textsuperscript{20}; pH in the asphyxia and asphyxia+TH+MLT groups was significantly different (p=0.044), but other parameters including duration of insult, and nadir of blood pressure and heart rate, and fetal oxygen saturation were not different between the asphyxia groups.

**Hypothermia**

TH lambs reached target temperature of 35°C within ~2.5h, and remained within 35±0.5°C circa 70% of the time, Table 1. TH lambs were rewarmed over 10h, and then remained stable at a core temperature of > 38.5°C over the remaining experimental period.

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Melatonin

MLT concentration was assessed during the experimental period in plasma, and in cortical brain tissue after post mortem at 72 h, Figure 1A & B. MLT concentration was not different between groups prior to asphyxia or MLT administration. As expected, iv. MLT administration significantly increased MLT concentration; in the control+MLT group, from baseline plasma MLT 0.08 ± 0.04 ng/ml to 425 ± 172 ng/ml at 12 h (p<0.001). Interestingly, circulating MLT concentrations were greater in the asphyxia+TH+MLT group than in the control+MLT and asphyxia+MLT groups (p<0.001). This difference was most notable at 48 h, when circulating levels were returning to baseline in both control+MLT and asphyxia+MLT cohorts (p<0.01, 24 h vs. 48 h) whereas the asphyxia+TH+MLT lambs showed no decrease in MLT concentrations from 24 h to 48 h (p=0.79). Brain concentrations of MLT were significantly elevated at 72 h in MLT-treated animals compared to asphyxia alone, but were not different in the asphyxia+TH+MLT and asphyxia+MLT groups (p=0.68).

Maintenance

By 4h after birth, all animals demonstrated stable physiological parameters (heart rate, $\text{SaO}_2$, base excess and lactate), Table 1. At 12h, asphyxia lambs showed secondary increase in base excess (p=0.01) and lactate (p=0.01) compared to control. Within the asphyxia groups, 2-way ANOVA showed that TH prevented the secondary increase in lactate (p=0.002 at 24h), but MLT did not (p=0.95), and there was no interaction between interventions (p=0.08).

Behavioral milestones

Control+MLT lambs showed behavioral outcomes expected for normal lambs but spent more time sleeping over the first 24h (control: 37%; control+MLT 63%; p=0.04), Table 2. Time to establish spontaneous feeding was delayed in asphyxia compared to the control groups (p=0.002). MLT treatment reduced the time taken for lambs to feed (p<0.001), whereas TH increased the time taken to feed (p=0.004), and there was no significant interaction between the treatments (p=0.06). At 72h, all control lambs could stand unaided. 11/16 asphyxia lambs could stand, but they took ~9-times longer than control lambs to attain standing position (p<0.0001). TH further increased the time to stand (p=0.001, asphyxia vs asphyxia+TH). Overt clinical seizures were present in 7/16 asphyxia lambs (vs 0/15 control, p=0.04). MLT treatment was associated with decreased seizures (p=0.03 vs asphyxia) but TH was not (p=0.16), with no interaction.

Magnetic resonance spectroscopy

Asphyxia alone was associated with a >2-fold increase in Lactate:Choline ratio (p=0.04 vs controls), a decrease in NAA:Choline (p=0.005), and 4-fold increase in Lactate:NAA (p=0.003), Figure 2. Two-
way ANOVA in the asphyxia groups showed that Lactate:Choline ratio was not significantly altered by MLT (p=0.13) or TH (p=0.49) and there was no interaction between variables (p=0.38).

NAA:Choline after asphyxia was not altered by MLT (p=0.11) or TH (p=0.13) but there was an interaction between TH and MLT (p=0.03), such that the asphyxia+TH+MLT group was not significantly different from controls. Lactate:NAA ratio after asphyxia was significantly reduced with MLT (p=0.008) but not TH (p=0.57), with no interaction (p=0.36). The asphyxia+TH+MLT lambs had metabolite concentrations that were not significantly different from control levels for all cerebral metabolic outcomes.

**Oxidative stress**

Cerebral oxidative stress was measured as MDA in CSF at post mortem (Figure 3A) and 4-HNE in brain tissue, Figure 3B, 6A-E. MDA was elevated 3-fold in CSF from asphyxia lambs versus control (p=0.02). Both MLT (p<0.0001) and hypothermia (p=0.0004) were associated with reduced MDA in CSF, with no interaction (p=0.45) and the combined treatment group asphyxia+TH+MLT was significantly reduced compared to asphyxia alone (p<0.0001) and asphyxia+TH (p=0.03). 4-HNE cell counts were not affected by asphyxia, but MLT significantly reduced 4-HNE cells within the thalamus only (p=0.006; Figure 3B), and TH had no affect.

**Inflammation**

IL-1β was the only cytokine that showed an appreciable increase within the CSF at 72h after asphyxia in asphyxia alone animals, Figure 4A. This increase in IL-1β was not present in any of the treatment groups. Neuroinflammation was assessed via IBA-1 cell counts, Figure 4B, 6F-J. Asphyxia increased IBA-1+ microglia within the cortex, hippocampus, striatum and white matter (p<0.05 vs control). Across the asphyxia groups, TH significantly reduced IBA-1 cell counts within all grey matter brain regions (p<0.01, 2-way ANOVA), with no significant effect in white matter. Similarly, MLT reduced IBA-1 in all brain regions (p<0.05), but with no significant effect in the striatum. There was no interaction between treatments for any brain region.

**Cell death**

S100B is a marker of neuronal and astrocyte damage. S100B levels were increased in CSF >5-fold following asphyxia (p=0.002 vs control, Figure 5A). Within the asphyxia groups, MLT treatment decreased S100B (p<0.0001), but TH did not (p=0.46). Post-hoc analysis suggested that both the asphyxia+MLT and asphyxia+TH+MLT groups showed a significant decrease in S100B compared to asphyxia alone. No treatment interaction was present (p=0.1).

Degenerating neurons stained with cresyl violet-acid fuchsin and morphological evidence of necrosis were counted (Figure 5B, 6K-O). Asphyxia alone was associated with a >2-fold increase in necrotic cell counts (p<0.001 for all regions vs control). Within the asphyxia groups, 2-way ANOVA showed
that TH was associated with a reduction in neuronal necrosis within the hippocampus, thalamus and striatum (p<0.05) and MLT reduced necrosis across all brain regions (p<0.001), with no significant interaction. On post-hoc analysis, neuronal necrosis was reduced in the asphyxia+TH group compared to asphyxia in the hippocampus only (p<0.05), while asphyxia+MLT was different to asphyxia across all brain regions (p<0.05). Furthermore, post-hoc analysis showed that neuronal degeneration was reduced to a greater degree in the asphyxia+TH+MLT group than asphyxia+TH within hippocampus, thalamus and striatum (p<0.05; Figure 5B).

Apoptosis was assessed using activated caspase-3 immunoreactivity and quantified as % area stained (Figure 5C, Figure 6P-T). A significant increase in activated caspase-3-area was observed in all brain regions after asphyxia (Figure 5C, 6P-T). Within the asphyxia groups, 2-way ANOVA showed that TH reduced activated caspase-3-area in all brain regions (p≤0.0001 for cortex, thalamus, striatum and white matter, and p=0.002 for hippocampus), while MLT treatment was associated with a significant reduction in activated caspase-3-area across all brain regions (p<0.05), except the cortex (p=0.065). MLT augmented the anti-apoptotic effects of TH within the thalamus and striatum, as shown by a significant treatment interaction (p<0.05). On post-hoc analysis, activated caspase-3-area was reduced in each of the asphyxia+TH, asphyxia+MLT and asphyxia+TH+MLT groups compared to asphyxia alone.

**DISCUSSION**

This study used a translational approach in lambs to demonstrate that TH and melatonin independently mediate neuroprotection after asphyxia and delivery by cesarean section. The addition of melatonin to therapeutic hypothermia additively augmented neuroprotection, with independent effects of TH and MLT to reduced inflammation, oxidative stress and both necrotic and apoptotic cell death. The combination of MLT i.v., commenced 30 minutes after asphyxia at birth, together with whole body TH started at 4 hours, improved neuro-behavioral outcomes, improved brain metabolic and biochemical profiles and reduced cell death. Results of this study confirm previous evidence that early initiation of MLT treatment is neuroprotective for perinatal asphyxia\(^{11}\) and that MLT augments hypothermic neuroprotection\(^{12,13,1,21-23}\). Here we extend these findings to show that the benefits of combined TH with MLT following birth asphyxia in lambs are greater than when either treatment is used in isolation across multiple indices of brain structure and function, including clinical markers of cerebral metabolism on MRS, and for caspase-3-mediated apoptosis.

Therapeutic hypothermia is the gold standard for treatment of neonatal encephalopathy, based on preclinical and clinical evidence that cooling reduces the progression of neuropathology and improves functional outcomes\(^{2,24,25}\). In the present study, TH was commenced at 4 hours after birth asphyxia in lambs using ice packs, as per the ICE trial\(^{26}\), and lambs were cooled for 24 hours to 35°C to reduce

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temperature by \(\sim 3.5\pm 0.5^\circ\text{C}\) compared to sham control lambs. We continued TH for 24 hours to align with evidence in piglets that this is feasible and neuroprotective\(^{27}\). Our results confirm that whole body cooling by 3.5\(^{\circ}\text{C}\) for 24 hours independently prevents a secondary increase in circulating lactate, neuroinflammation and cerebral apoptosis, with a brain-region specific reduction in necrotic cell death, consistent with previous preclinical experimental studies\(^{28-30}\). By contrast, in the present study TH did not improve the lactate:NAA ratio on MRS, whereas previous clinical assessment supports that TH improves this measure\(^{31}\). We acknowledge that this could reflect that the duration of TH was not sufficient for maximal neuroprotection in the current study, where 72 hours of TH in fetal sheep consistently provides optimal effect\(^{32}\). Nevertheless, TH provided independent neuroprotection across multiple outcome measures, confirming that this protocol provides a robust basis to test adjuvant therapies to augment neuroprotection with TH after perinatal asphyxia.

MLT infusion resulted in a rapid and sustained increase in circulating MLT levels, and brain concentrations of MLT at 72h (48h after the final MLT administration) were elevated in MLT groups. Interestingly, circulating MLT levels remained elevated in asphyxia+TH+MLT lambs compared to the asphyxia+MLT group. This may reflect suppression of basal metabolic rate and drug clearance by TH\(^{33}\), reducing liver metabolism and renal excretion of MLT. This prolonged elevation in circulating MLT may have contributed to the greater neuroprotection seen in the asphyxia+TH+MLT group compared to other treatment groups, although brain MLT levels at 72 hours were not different across MLT treated groups. We have previously shown that even a smaller increase in cerebral MLT is neuroprotective\(^{11}\). MLT administration was associated with independent neuroprotective effects, including reduced cerebral lipid peroxidation production and, at least regionally, decreased oxidative stress within the thalamus\(^{34}\). MLT was also associated with anti-inflammatory and anti-apoptotic effects across many brain regions, and reduced neuronal degeneration across all brain regions. A number of these effects of MLT were similar to actions of TH, but interestingly, MLT but not TH was associated with reduced cerebral Lactate:NAA at 72h and MLT reduced the cerebral injury marker S100B in CSF whereas TH did not.

In this study we utilized a modified Sarnat grading system to assess neonatal lamb wellbeing and time to achieve typical milestones that consider feeding ability, standing unaided and physical presence of seizures-like activity\(^{15,16}\). As expected, asphyxia lambs showed abnormalities in all of these milestones. The results show that 100% of asphyxia+TH+MLT animals were able to stand independently and feed from a bottle at 72 hours, and the time taken to achieve feeding and standing were significantly reduced compared to TH alone. Furthermore, it is notable that none of the lambs in the asphyxia+TH+MLT group exhibited physical signs of seizure-like activity, compared to 44% in the asphyxia alone group, suggesting that MLT augments the effects of TH to prevent seizures. Many neonatal seizures in NE are clinically silent, and so this result requires confirmation with EEG monitoring. Thus, co-treatment of MLT with TH improved neurological recovery compared to both
asphyxia alone and asphyxia+TH. These marked improvements in functional outcomes are an excellent sign for clinical applicability.

Increased Lactate:NAA and/or a decrease in NAA (either NAA:Choline or NAA:Creatine) in the neonatal period following perinatal asphyxia are highly predictive for death or major disability at 12-24 months\textsuperscript{35,36}. In the current study, Lactate:Choline, NAA:Choline and Lactate:NAA were all significantly affected at 72h after asphyxia (Figure 2), demonstrating cerebral metabolic disturbance and reduced neuronal wellbeing\textsuperscript{37}. Following asphyxia, MLT treatment but not TH was independently associated with improved Lactate:NAA ratio. It was interesting that neither TH or MLT independently improved NAA:Choline, but that combination treatment was associated with a significant interaction effect, such that NAA:Choline values in the asphyxia+TH+MLT animals were improved and not different from controls. Our data reveal a discrepancy between the lack of change in NAA:Choline as a measure of neuronal function in the deep gray matter of asphyxia+MLT and asphyxia+TH groups, compared with reduced histological cellular necrosis and apoptosis in these groups. A reduction in brain NAA is considered a sensitive marker for reduced neuronal metabolism, but there remains conjecture regarding whether this is reversible or irreversible\textsuperscript{38}. Our results suggest that both MLT and TH independently reduced neuronal necrosis and apoptosis, but that these cell populations may not be fully functional at 72 hours. Critically, for both the MRS measure of NAA:Choline and apoptotic assessment of cell death our results strongly support the concept that MLT augments the neuroprotective action of TH to maintain neuronal wellbeing following severe asphyxia.

Neuronal loss following a severe perinatal asphyxic insult is mediated via both apoptotic and necrotic cell degeneration\textsuperscript{19}. MLT exhibited strong neuroprotective benefit to reduce necrosis across all brain regions, while TH reduced neuronal necrosis only in the deep grey matter brain regions of the thalamus, striatum and hippocampus. Conversely, TH was strongly anti-apoptotic across all brain regions, whereas the effects of MLT were region-specific. These observations support previous work in newborn infants that TH has a greater effect to suppress apoptotic cell death versus necrotic cell death\textsuperscript{39,40} and suggests that MLT prevents necrosis to a greater extent than apoptosis. Neuronal death following severe asphyxia is a continuum and caspase-3-dependent apoptosis persists from days to weeks to mediate neonatal encephalopathy\textsuperscript{41}. An interaction was observed between TH and MLT for anti-apoptotic benefit within the deep grey matter of the thalamus and striatum (Figure 5B), indicating that MLT augments TH to reduce apoptosis to a greater degree than either treatment alone.

Perinatal asphyxia initiates profound cerebral oxidative stress and inflammatory responses, which are critical mediators of neuronal degeneration\textsuperscript{42}. MLT and TH can both prevent mitochondrial dysfunction\textsuperscript{43,44}, and MLT is a potent direct scavenger of ROS\textsuperscript{45}. Therefore it is not surprising that in the current study we show that cerebral lipid peroxidation products (MDA in CSF and cellular 4-HNE) were reduced in the asphyxia+MLT and asphyxia+TH lambs compared to asphyxia alone, albeit antioxidant effects for both TH and MLT were partial and region-specific (Figure 3). Strikingly, we
show that the combination of MLT and TH has substantial antioxidant benefits in response to asphyxia, with the lipid peroxide product MDA significantly reduced in the asphyxia+TH+MLT group compared to asphyxia+TH. The combined treatment group had the lowest levels of cellular lipid peroxidation across all groups and brain regions. Similarly, the present study confirms that TH and MLT independently reduce cellular neuroinflammation. The present study supports independent actions of TH and MLT, with no interactions between the two interventions, but that the effects of the combined treatments were generally broader and more pronounced; for example, only the asphyxia+TH+MLT group had reduced neuroinflammation compared to asphyxia alone in all grey matter regions.

We aimed to use a clinically applicable model of perinatal asphyxia and neonatal care and monitoring; however, some limitations should be considered. Firstly, it will be challenging to clinically administer MLT within the first hour after birth and 2-hourly IV administration would be onerous during neonatal intensive care. A continuous i.v. infusion may be possible. Alternatively, intragastric administration of melatonin in preterm and term human infants, has been associated with sustained melatonin concentrations with reduced frequency of administration compared to animal studies. We also found that early administration of MLT made normal lambs sleepier than controls, and this sleepiness may mask the encephalopathic symptoms that guide the requirement for TH. Finally, MLT was dissolved in ethanol prior to intravenous administration. We did not include a control or asphyxia group with vehicle (ethanol) only. Others have shown that ethanol itself may mediate brain-region specific neuroprotection within the developing brain. We do however note that Robertson and colleagues are exploring an ethanol-free MLT formulation and this will be critical for the translation of this work.

It also should be noted that lambs in TH groups (asphyxia+TH and asphyxia+TH+MLT) spent 70% of time at the goal cooled temperature of 35±0.5°C, and, combined with a reduced 24 hour period of TH compared to the clinical situation would likely contribute to reduced efficacy of hypothermia treatment. Our results support that both TH and MLT and combined treatments are safe. The only potential adverse effects were a tachycardic response to TH at 12 h, and sedative effects of MLT and TH. TH is now routine clinical care for neonatal encephalopathy. Although MLT is not used routinely in neonatal care, it has been administered to newborns without adverse effects.

This study supports that MLT has additive neuroprotective benefits when used together with TH for neonatal encephalopathy. Importantly, TH and MLT together had significant additive benefits, such that outcomes were better than when either TH or MLT was given in isolation. We observed a significant interaction between TH and MLT to restore functional outcomes and normalization of neuronal function (NAA) on MRS. Critically, 100% of asphyxia+TH+MLT lambs were able to stand and feed, in contrast with only partial recovery in the TH and MLT groups. Although both TH and MLT independently showed antioxidant and anti-inflammatory properties, there were no apparent interactions between these outcomes, suggesting that other mechanisms contributed to the combined

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benefits. Interestingly, the cellular effects of TH and MLT appear to be region-specific within the brain, and, in co-treated group showed more consistent neuroprotection across multiple regions. A significant treatment interaction within the deep gray matter for caspase-3-mediated apoptosis indicates that MLT augments the neuroprotective benefits of TH to reduce ongoing cell death. These findings confirm the neuroprotective efficacy of TH in a lamb model of perinatal asphyxia and demonstrate that MLT provides additive neuroprotective benefit. These results will guide future trials of combined treatments to improve outcomes for infants with neonatal encephalopathy.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance of Jan Loose, Yen Pham, Courtney McDonald, Domenic LaRosa, Stacey Ellery, Jingang Li, Madison Paton, Robert Alers, Lesley Wiadrowski, David Shipp, Richard McIntyre, and Patricia Heidmann.

FUNDING

This study was supported by the National Health and Medical Research Council (NHMRC) of Australia, the Victorian Government’s Operational Infrastructure Support Program, and the Bill and Melinda Gates Foundation.
REFERENCES


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Table 1. Lamb physiological outcomes. Significantly different values indicted in bold. Data are mean ± standard error of the mean. *p<0.05 vs control; #p<0.05 vs asphyxia; ɸp<0.05 vs asphyxia+MLT.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control+MLT</th>
<th>Asphyxia</th>
<th>Asphyxia+TH</th>
<th>Asphyxia+MLT</th>
<th>Asphyxia+TH+MLT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In-utero</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number (Male/Female)</td>
<td>9 (5/4)</td>
<td>6 (1/5)</td>
<td>16 (6/10)</td>
<td>12 (6/6)</td>
<td>14 (8/6)</td>
<td>6 (3/3)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>199 ± 11</td>
<td>194 ± 14</td>
<td>183 ± 6</td>
<td>226 ± 15</td>
<td>197 ± 14</td>
<td>213 ± 18</td>
</tr>
<tr>
<td>SaO₂ (%)</td>
<td>68.1 ± 3.8</td>
<td>68.9 ± 3.5</td>
<td>69.0 ± 3.6</td>
<td>72.3 ± 2.9</td>
<td>73.9 ± 1.9</td>
<td>75.7 ± 3.3</td>
</tr>
<tr>
<td>pH</td>
<td>7.26 ± 0.02</td>
<td>7.28 ± 0.01</td>
<td>7.23 ± 0.02</td>
<td>7.29 ± 0.02</td>
<td>7.24 ± 0.01</td>
<td>7.31 ± 0.03</td>
</tr>
<tr>
<td>Base excess (mmol/L)</td>
<td>-0.5 ± 0.9</td>
<td>-0.1 ± 0.6</td>
<td>-2.1 ± 1.2</td>
<td>-2.0 ± 0.8</td>
<td>-0.2 ± 0.3</td>
<td>-0.9 ± 0.9</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>2.2 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td><strong>Asphyxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCO duration (min)</td>
<td>-</td>
<td>-</td>
<td>9.9 ± 0.5</td>
<td>10.3 ± 0.4</td>
<td>9.7 ± 0.3</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td>SaO₂ (%)</td>
<td>-</td>
<td>-</td>
<td>5.8 ± 2.3</td>
<td>5.5 ± 1.5</td>
<td>5.5 ± 1.5</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>-</td>
<td>6.87 ± 0.02</td>
<td>6.92 ± 0.02</td>
<td>6.91 ± 0.02</td>
<td>6.96 ± 0.02</td>
</tr>
<tr>
<td>Base excess (mmol/L)</td>
<td>-</td>
<td>-</td>
<td>-13.9 ± 1.1</td>
<td>-14.0 ± 0.6</td>
<td>-13.4 ± 0.9</td>
<td>-12.5 ± 1.3</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>-</td>
<td>-</td>
<td>9.4 ± 0.6</td>
<td>7.5 ± 0.5</td>
<td>8.9 ± 0.4</td>
<td>7.3 ± 0.8</td>
</tr>
<tr>
<td><strong>Resuscitation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenaline use</td>
<td>0% (0/9)</td>
<td>0% (0/6)</td>
<td>25% (4/16)</td>
<td>42% (5/12)</td>
<td>29% (4/14)</td>
<td>33% (2/6)</td>
</tr>
<tr>
<td>Spontaneous breathing (min)</td>
<td>10 ± 3</td>
<td>53 ± 28</td>
<td><strong>669 ± 383</strong></td>
<td>351 ± 296</td>
<td>25 ± 4</td>
<td>59 ± 16</td>
</tr>
<tr>
<td>Ventilation time (min)</td>
<td>21 ± 3</td>
<td>72 ± 34</td>
<td><strong>694 ± 381</strong></td>
<td>800 ± 475</td>
<td>78 ± 18</td>
<td>101 ± 21</td>
</tr>
<tr>
<td><strong>Temperature (℃)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to goal temperature (h)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.7 ± 0.6</td>
<td>-</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Time at goal (%)</td>
<td>-</td>
<td>-</td>
<td>67 ± 5</td>
<td>-</td>
<td>74 ± 8</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>38.6 ± 0.2</td>
<td>38.7 ± 0.1</td>
<td>38.6 ± 0.1</td>
<td>38.0 ± 0.3</td>
<td>38.8 ± 0.3</td>
<td>37.8 ± 0.7</td>
</tr>
<tr>
<td>12 h</td>
<td>38.3 ± 0.4</td>
<td>37.9 ± 0.2</td>
<td>38.0 ± 0.3</td>
<td><strong>35.2 ± 0.1</strong></td>
<td>37.7 ± 0.4</td>
<td><strong>35.9 ± 0.4</strong></td>
</tr>
<tr>
<td>24 h</td>
<td>38.2 ± 0.2</td>
<td><strong>39.1 ± 0.1</strong></td>
<td>38.5 ± 0.2</td>
<td><strong>34.9 ± 0.1</strong></td>
<td>38.6 ± 0.1</td>
<td><strong>35.2 ± 0.2</strong></td>
</tr>
<tr>
<td>48 h</td>
<td>38.4 ± 0.3</td>
<td><strong>39.5 ± 0.1</strong></td>
<td>38.8 ± 0.1</td>
<td>38.5 ± 0.2</td>
<td>38.4 ± 0.2</td>
<td>38.8 ± 0.3</td>
</tr>
<tr>
<td>72 h</td>
<td>38.7 ± 0.3</td>
<td>39.3 ± 0.2</td>
<td>39.0 ± 0.1</td>
<td>39.0 ± 0.1</td>
<td>38.7 ± 0.2</td>
<td><strong>39.6 ± 0.2</strong></td>
</tr>
</tbody>
</table>
Table 2. Lamb functional outcomes. Significantly different values are indicated in bold. Data are mean ± standard error of the mean. *p<0.05 vs control; #p<0.05 vs asphyxia; ^p<0.05 vs asphyxia+TH.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control+MLT</th>
<th>Asphyxia</th>
<th>Asphyxia+TH</th>
<th>Asphyxia+MLT</th>
<th>Asphyxia+TH+MLT</th>
</tr>
</thead>
</table>
Fig. 1. Melatonin (MLT) concentration. MLT concentrations in plasma (A) were significantly increased in control+MLT, asphyxia+MLT and asphyxia+TH+MLT over the experimental period. MLT concentration showed a prolonged increase in asphyxia+TH+MLT animals. MLT concentration within the cortex (B) was significantly elevated in all MLT-treated groups. Data are mean ± standard error of the mean. The results from 2-way ANOVA amongst asphyxia groups are shown on the figure. *p<0.05 vs control; #p<0.05 vs asphyxia; ϕp<0.05 vs asphyxia+TH; ϕϕp<0.05 vs asphyxia+MLT.
Fig. 2. Magnetic resonance spectroscopy (MRS). Lactate:Choline (A) was significantly increased in the asphyxia group compared to control. There were no significant differences following 2-way ANOVA among asphyxia groups. NAA:Choline (B) showed a significant deficit in the asphyxia group compared to control, indicating neuronal impairment. 2-way ANOVA within asphyxia groups revealed a significant interaction between TH and MLT (p=0.03). Lactate:NAA (C) was increased in the asphyxia group compared to control. 2-way ANOVA within asphyxia groups showed a significant effect with MLT treatment (p=0.008). Data presented as mean ± standard error of the mean. The results from 2-way ANOVA amongst asphyxia groups are shown on the figures. *p<0.05 vs control.
Fig. 3. Markers of cerebral oxidative stress. Malondialdehyde (MDA) concentration (A) within cerebrospinal fluid (CSF) was significantly increased in asphyxia animals compared to control. 2-way ANOVA across asphyxia groups show that both therapeutic hypothermia (TH) and melatonin (MLT) significantly reduced MDA concentration compared to asphyxia alone. (B) 4-hydroxynonenal (4-HNE; log scale) cell counts from the cortex (molecular layer), hippocampus (CA1), thalamus (paraventricular nuclei), striatum (external capsule), and subcortical white matter. 4-HNE-positive cells were not significantly different from controls after asphyxia. 2-way ANOVA across asphyxia groups showed that MLT decreased 4-HNE count within the thalamus only and TH did not affect 4-HNE cell counts. Data presented as mean ± standard error of the mean. The results from 2-way
Fig. 4. Markers of cerebral inflammation. IL-1β concentration (A) within cerebrospinal fluid (CSF) was significantly increased following asphyxia compared to control. (B) Ionized calcium binding adapter molecule (IBA-1) cell counts within the cortex (molecular layer), hippocampus (CA1), thalamus (paraventricular nuclei), striatum (external capsule), and subcortical white matter. Asphyxia significantly increased IBA-1 cell counts in all brain regions except the thalamus compared to control. 2-way ANOVA across asphyxia groups showed that TH reduced IBA-1 cell counts within all brain regions except the white matter and MLT reduced counts in all regions except the striatum. Data presented as mean ± standard error of the mean. The results from 2-way ANOVA amongst asphyxia groups are shown on the figures. *p<0.05 vs control; #p<0.05 vs asphyxia.
Fig. 5. Markers of cerebral cell death. S100B concentration (A) within the cerebrospinal fluid (CSF) was significantly increased in asphyxia-only animals compared to control. Across asphyxia groups, MLT treatment was associated with a decrease in CSF S100B, but TH was not. (B) Neurons stained with cresyl violet-acid fuchsin and demonstrating evidence of cellular necrosis were counted and (C) activated caspase-3 % area staining for apoptosis within the cortex (molecular layer), hippocampus (CA1), thalamus (paraventricular nuclei), striatum (external capsule), and subcortical white matter. Asphyxia was associated with a significant increase in cellular necrosis and apoptosis in all brain regions compared to control. 2-way ANOVA across asphyxia groups showed that TH reduced neuronal necrosis within the hippocampus, thalamus and striatum and...
reduced apoptosis across all regions, while MLT reduced both necrosis and apoptosis in all brain regions. Data are mean ± standard error of the mean. The results from 2-way ANOVA amongst asphyxia groups are presented on the figure. *p<0.05 vs control; #p<0.05 vs asphyxia; †p<0.05 vs asphyxia+TH.

**Fig. 6.** Photomicrographs from brain tissue collected at 72 h after birth. Representative images are from the striatum (external capsule) for staining with 4-hydroxynonenal (4-HNE; A-E), ionized calcium binding adapter molecule (IBA-1; F-J), cresyl violet-acid fuchsin (CVAF; K-O) and activated caspase-3 (Cas-3; P-T). White arrows indicate cells showing evidence of necrosis; scale bar = 100 µm.
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Aridas, JDS; Yawno, T; Sutherland, AE; Nitsos, I; Wong, FY; Hunt, RW; Ditchfield, M; Fahey, MC; Malhotra, A; Wallace, EM; Gunn, AJ; Jenkin, G; Miller, SL

Title:
Melatonin augments the neuroprotective effects of hypothermia in lambs following perinatal asphyxia

Date:
2021-06-20

Citation:

Persistent Link:
http://hdl.handle.net/11343/298658