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Early Antioxidant Treatment and Delayed Hypothermia After Hypoxia-Ischemia Have No Additive Neuroprotection in Newborn Pigs

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Abstract

Background—The implementation and clinical efficacy of hypothermia in neonatal hypoxic-ischemic (HI) encephalopathy are limited, in part, by the delay in instituting hypothermia and access to equipment. In a piglet model of HI, half of the neurons in putamen already showed ischemic cytopathology by 6 hours of recovery. We tested the hypothesis that treatment with the superoxide dismutase-catalase mimetic EUK-134 at 30 minutes of recovery provides additive neuronal protection when combined with one day of whole body hypothermia implemented 4 hours after resuscitation.

Methods—Anesthetized piglets were subjected to 40 minutes of hypoxia (10% inspired oxygen) followed by 7 minutes of airway occlusion and resuscitation. Body temperature was maintained at 38.5°C in normothermic groups and at 34°C in hypothermic groups. All groups were mechanically ventilated, sedated, and received muscle relaxants during the first day of recovery. Neuropathology was assessed by profile and stereological cell counting methods.

Results—At 10 days of recovery, neuronal viability in putamen of a normothermic group treated with saline vehicle was reduced to 17±6% (±95% confidence interval) of the value in a sham-operated control group (100±15%). Intravenous infusion of EUK-134 (2.5 mg/kg at 30 minutes of recovery + 1.25 mg/kg/h until 4 hours of recovery) with normothermic recovery resulted in 40±12% viable neurons in putamen. Treatment with saline vehicle followed by delayed hypothermia resulted in partial protection (46±15%). Combining early EUK-134 treatment with delayed hypothermia also produced partial protection (47±18%) that was not significantly greater than single treatment with EUK-134 (confidence interval of difference: −15% to 29%) or delayed hypothermia (−16% to 19%). Furthermore, no additive neuroprotection was detected in caudate nucleus or parasagittal neocortex, where neuronal loss was less severe.

Conclusions—We conclude that early treatment with this antioxidant does not substantially enhance the therapeutic benefit of delayed hypothermia in protecting highly vulnerable neurons in HI-insulted newborns, possibly because basal ganglia neurons are already undergoing irreversible cell death signaling by the time EUK-134 is administered or because this compound and hypothermia attenuate similar mechanisms of injury.

Introduction

Neonatal hypoxic-ischemic (HI) encephalopathy is a clinically significant disorder with long-term morbidity. Therapeutic hypothermia has been used to limit brain damage in term newborns, but efficacy in improving neurological outcome is incomplete.^{1–5} One factor that likely contributes to incomplete efficacy is the delay in inducing hypothermia after the insult. Most newborns enrolled in hypothermic clinical trials of HI encephalopathy had hypothermia induced between 3 and 6 hours after birth.^{1,2,4} In experimental studies, hypothermia is neuroprotective when induced immediately after HI in neonatal rodents⁶ and piglets^{7–9} and when induced 1.5 hours after HI in fetal sheep.¹⁰ Studies in animals show that

the efficacy of hypothermia is diminished when induction is delayed by several hours. In fetal sheep, efficacy of hypothermia seen with a 5.5-hour delay after HI¹¹ was lost when induction was delayed by 8.5 hours.¹² In a piglet model of severe HI, neuroprotection from hypothermia was lost when the onset was delayed by 3 hours.¹³ Therefore, a clinical need remains for finding agents that can be administered easily and safely after birth and that can provide an added benefit with delayed hypothermia in protecting the brain from HI.

Basal ganglia, thalamus, and primary sensorimotor cortex are particularly vulnerable to HI in term newborns, and those with basal ganglia and thalamic damage are likely to have poor outcome and severe motor deficits.^{14–16} Induction of moderate hypothermia in term newborns is capable of reducing lesions in basal ganglia and thalamus.^{17,18} In newborn piglets exposed to hypoxia followed by asphyxia, neurodegeneration in striatum precedes that in other regions, and cell death is particularly prominent in putamen.^{19,20} Oxidant stress is considered a major contributing factor to neuronal damage after HI in immature brain,²¹ and various markers of oxidative stress are evident in piglet striatum at 3 and 6 hours of recovery. These markers include decreased glutathione concentration, increased formation of protein carbonyl groups, nitration of proteins and nucleic acids, and hydroxylation of nucleic acids.^{20,22,23} Disruption of the Golgi apparatus over the first 6 hours precedes neuronal cell death that occurs progressively between 6 and 24 hours in piglet striatum.²² Therefore, alleviating early oxidant stress in striatum may sustain neuronal viability until delayed hypothermia can be instituted to provide more robust neuroprotection.

We previously found that IV administration of EUK-134 (manganese 3-methoxy N,N'-bis(salicylidene)ethylenediamine chloride) to piglets at 30 minutes of recovery from HI reduced markers of oxidative stress and provided partial protection of neurons in putamen and caudate nucleus.²⁴ This drug is a member of the manganese-salen family of catalytic antioxidants that remove superoxide and hydrogen peroxide and oxidize nitric oxide and peroxynitrite.^{25–27} Although neuroprotection evaluated at 4 days of recovery was only partial when EUK-134 was administered at 30 minutes after HI, significant reductions in protein carbonyl and nitrotyrosine formation were observed at 3 hours of recovery.²⁴ Thus, this agent has the potential to sustain neuronal viability until they can be fully rescued by delayed hypothermia. Induction of hypothermia 5 minutes after resuscitation from hypoxia plus asphyxia in piglets is able to suppress early oxidative stress and completely block striatal neuron loss determined 10 days after rewarming.^{7,23} However, we anticipated that delaying hypothermia until 4 hours of recovery in this model would provide only partial protection in striatum because of the marked oxidative stress and organelle disruption that are already present at 3–6 hours of recovery.²² Therefore, in the present study we tested the hypothesis that combining early EUK-134 treatment at 30 minutes after HI with 1 day of hypothermia induced at 4 hours after HI would result in additive striatal neuroprotection compared to individual treatments.

Methods

Animal preparation

All procedures on piglets were approved by the Animal Care and Use Committee at the Johns Hopkins University. Animal preparation was performed as previously described.²⁸<http://ovidsp.tx.ovid.com/spb/ovidweb.cgi?>

QS2=434f4e1a73d37e8c04d9a87dbb68790670a0e070fd416baef874d8ddef8eb2a525de4b05856410d19fde3aaa92a031ef44e1f0404db083046e82bf13145b777e717fda5472637eb93dd2a562ac8b0c036b84cb9fa8755b6713ac1ba9b7f4b0772141f6f4c8226499d5fab27280c1621bea9343c25e3f78f575635bafd87bf0ac66bad78113fd8f454f958062144e87df9217b5916fe13973ea7f0b8102273270efd6e4dca03455d18e8f6c27ef3fc3be489e1e77d852cfbfc25ecd4f46e2b9fd030d0476a38581549666512b38b365cefd1e451b75744ccadf2235b

84a8e4efb2bf4c763f3703aa8fb8 - 90#90 Three-seven-day-old male piglets, weighing 1.5–3.0 kg, were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal), tracheally intubated, and their lungs mechanically ventilated to maintain normoxia and normocarbida. Normal rectal temperature during the preparation was maintained at $38.5 \pm 0.5^\circ\text{C}$ by heating blankets and overhead lamps. Under aseptic conditions, catheters were inserted into a femoral arterial and vein through an incision in the groin, tunneled subcutaneously, and secured to a flank exit incision. After catheterization, fentanyl (20 $\mu\text{g/kg}$) was injected IV, and 5% dextrose in 0.45% saline was continuously infused at 10 mL/h. Cephalothin (100 mg IV) was given as a prophylactic antibiotic daily for 3 days.

Experimental protocol

Mean arterial blood pressure, heart rate, pulse oximetry, end-tidal CO_2 , and rectal temperature were continuously monitored for 24 hours. Pancuronium (0.3 mg/kg) was injected IV to prevent respiratory muscle movement during hypoxia and asphyxia. Hypoxia was produced for 40 minutes by decreasing inspired O_2 to 9.8%.²⁸ After hypoxia, the piglets' lungs were briefly ventilated with 21% O_2 for 5 minutes before the airway was occluded for 7 minutes. This procedure resulted in severe hypotension, bradycardia, and sometimes asystole. It was necessary to briefly reoxygenate the piglets before asphyxia to achieve a high rate of successful cardiac resuscitation. After 7 minutes of asphyxia, we performed cardiopulmonary resuscitation by instituting pulmonary ventilation with 50% O_2 and, if necessary, chest compressions at a rate of 100/min and IV injections of epinephrine (0.1 mg/kg). If return of spontaneous circulation (ROSC) did not occur, a second injection of epinephrine was given; defibrillation (2–5 J/kg) was performed if ventricular fibrillation was evident on the electrocardiogram. Animals were excluded from further study if spontaneous circulation did not return within 3 minutes after the airway was unclamped. Arterial blood samples were obtained pre-arrest, at 5, 15, 25, and 37 minutes of hypoxia, at 4 minutes of room air ventilation, at 5 minutes of asphyxia, and at 0.1, 0.5, 1, 2, 3, 4, 5, 6, 9, 12, 15, 18, 21, and 24 hours after ROSC for measurements of partial pressure of O_2 (PaO_2) and CO_2 (PaCO_2), pH, arterial O_2 saturation, hemoglobin concentration, and glucose concentration. After ROSC, sodium bicarbonate was infused as needed to correct the base deficit, and inspired O_2 concentration was reduced to ~30% to maintain arterial O_2 saturation. Arterial pH, PCO_2 , and PO_2 were measured at 37°C and corrected to body temperature. Arterial pH was managed with a pH-stat strategy. Three hours after resuscitation, continuous IV infusions of fentanyl (10–30 $\mu\text{g/kg/h}$) and pancuronium (0.1–0.2 mg/kg/h) were started.⁷ Sedation and muscle paralysis were used to reduce the stress and shivering response to cooling, which have been shown to counteract the benefit of hypothermia in piglets.^{8,29} The dose of fentanyl was increased as needed for an increase in heart rate or arterial blood pressure. Piglets also received a continuous IV infusion of 5% dextrose in 0.45% saline at a rate of 10–15 mL/h for the first 24 hours of recovery. They also underwent repositioning every 2 hours, endotracheal suctioning every 4 hours, ocular lubrication, and a suprapubic bladder tap if needed.

After HI, piglets were divided into four treatment groups: normothermia plus saline vehicle, hypothermia plus saline vehicle, normothermia plus EUK-134, and hypothermia plus EUK-134. EUK-134 (Cayman Chemical, Ann Arbor, MI, USA), dissolved in normal saline, was infused IV with an initial loading dose of 2.5 mg/kg at 30 minutes after ROSC followed by a maintenance infusion of 1.25 mg/kg/h until 4 hours after ROSC. This dosing regimen is the same as that used previously in piglets²⁴ and similar to the regimen used to show protection from focal ischemia in adult rats.³⁰ In hypothermic groups, we initiated cooling at 4 hours after ROSC by applying cooling blankets (10°C) underneath the supine piglet and by applying ice packs around the head and torso. When rectal temperature reached 36°C , the ice packs were removed from the head. When rectal temperature reached 35°C , the ice was

removed from the torso. Target rectal temperature of 34°C was maintained by adjusting the temperature of the circulating-water blanket. With this method of cooling, brain temperature was found to closely track rectal temperature.⁷ Hypothermic piglets were rewarmed beginning at 20 hours after ROSC at a rate of approximately 1°C/h over 4 hours by adjusting the temperature of the cooling blanket until rectal temperature reached 38.5–39.0°C (normal for piglets). Between 20 and 24 hours after ROSC, the infusion rate of fentanyl and pancuronium was decreased incrementally every hour by 25% of the rate that was used at 20 hours. In normothermic animals, rectal temperature was maintained near a target temperature range of 38.5–39.0°C with a warming blanket and overhead heating lamps during the recovery time. Normothermic piglets were treated in the same manner as the hypothermic piglets with regard to the duration of fentanyl and pancuronium infusion and mechanical ventilation. Cohorts of surgical sham animals without HI exposure underwent the same anesthesia, surgery, 20 hours of sedation, muscle paralysis, and temperature manipulations as HI animals. These control cohorts were also divided into the same four treatment groups as the HI cohorts.

Piglets were tracheally extubated after recovery of ventilatory effort and were moved to a padded cage where they were observed, fed, and mobilized at 2–4-hour intervals during the second day and evening of recovery. All piglets continued to receive IV fluids and initially were syringe-fed reconstituted swine milk. When they were able to drink milk independently from a bowl, the IV fluid administration was stopped. After adequate recovery, animals ambulated, and were generally kept in a pigpen with other piglets during the day and housed with another piglet at night.

Histologic assessment

At 10 days after ROSC, piglets were deeply anesthetized with sodium pentobarbital (100 mg/kg intraperitoneally). Brains were perfused transcardially with ice-cold phosphate-buffered saline (PBS) until venous return was clear and then with ice-cold 4% paraformaldehyde prepared in PBS. Brains were removed and bisected mid-sagittally; each hemisphere was cut into 1-cm coronal slabs. A 3- to 4-mm-thick slice was taken from the forebrain at a mid-striatal level and embedded with paraffin. Neuronal damage in mid-striatal samples was quantified in 10- μ m sections stained with hematoxylin and eosin (H&E). For each piglet, the neuronal damage was determined by counting viable neuronal cell profiles in sections in seven non-overlapping fields in the putamen and caudate nucleus and in 10 fields in layer V of parasagittal neocortex. Cells were counted under oil immersion at 1000 $\times$ magnification with the use of a microscopic grid. An average value from the seven or 10 fields was obtained on one section for each animal. Profile counting was performed by an investigator blinded to treatment group.

In a subset of piglets used for the profile counting-based method, unbiased stereology was performed in putamen, where injury was most severe. The contralateral hemisphere was cryoprotected in 20% glycerol and cut into 50- μ m coronal sections that were stained with cresyl violet. Estimates of total remaining neurons were determined using disector frame areas of 4488 μ m² with a guard thickness of 2 μ m on every 12th section with the aid of Stereo Investigator Software (MBF Bioscience, Williston, VT). With approximately 90 counting frames sampled per putamen, the number of neurons counted in each sham piglet was approximately 400. Because some tissue blocks were unavailable, the entire putamen could not be counted in every brain, and results are expressed as a volume density.

Neurologic deficit score

The level of consciousness, brainstem function, sensory responses, motor function, spatial orientation, behavioral activity, and clinical seizure activity were scored as previously

described.⁷ The score was summed to obtain a composite score with a maximum deficit score of 154 and a normal score of 0.

Statistical analysis

Based on an average SD of 10% of the sham value in previous work for viable neuronal counts in putamen,²⁴ a sample size of 10 was targeted to provide detection of 20% relative differences between groups at 93% power for an unadjusted α of 0.01. Physiological data and neuronal counts passed the normality test ($P > 0.05$) and the homogeneity of variance test ($P > 0.05$). Comparisons among five groups (the four HI groups and the combined sham group) were made by using one-way analysis of variance. If the F -value was significant, the Holm-Sidak procedure was used to identify differences between groups with the family-wise error rate set at < 0.05 . For data that did not pass the normality test (neurologic deficit score and intensive care management variables), comparisons were made among groups by the Kruskal-Wallis analysis of ranks and Dunn's method for multiple comparisons. The level of statistical significance was set at corrected $P < 0.05$.

Results

Physiologic results

Sixty-two piglets were studied, including eight sham-operated piglets and 54 piglets that were subjected to HI. Resuscitation was successful in 46 of the HI piglets (85%). Two piglets required ventricular defibrillation. Two piglets died (one normothermic with saline, one normothermic with EUK-134) during the first 4 days as a result of severe seizures. Three piglets were euthanized (one normothermic with saline, two hypothermic with EUK) due to severe diarrhea. Long-term survivors with histological analysis included 11 piglets in the normothermic plus saline group, 10 piglets in the hypothermic plus saline group, 10 piglets in the normothermic plus EUK-134 group, and 10 piglets in the hypothermic plus EUK-134 group. There were no deaths among sham-operated piglets.

During the 40-minute period of hypoxia, a small degree of acidemia occurred with no change in PaCO_2 , whereas severe acidemia accompanied by severe hypercapnia developed by 5 minutes of asphyxia (Table 1). After resuscitation, pH, PaO_2 , and PaCO_2 recovered to baseline values within 60 minutes in each group and were similar to values in the sham groups (sham data not shown). Some small statistical differences in arterial pH and PaO_2 were detected among groups at 6 and 12 hours after resuscitation, but these were not considered physiologically significant. Hypoxia and asphyxia produced a transient hyperglycemic response that was highly variable and not significantly different among groups (Table 1).

During hypoxia, all groups had a similar decrease in arterial O_2 saturation, increase in heart rate, and moderate increase in mean arterial blood pressure (Figure 1), although pressure in the hypothermic plus saline group was significantly higher than in the normothermic plus EUK-134 group at 15 and 27 minutes of hypoxia. During asphyxia, arterial O_2 saturation, heart rate, and arterial blood pressure each decreased substantially. The duration of cardiopulmonary resuscitation and the cumulative dose of epinephrine were similar among groups (Table 2). After resuscitation, arterial O_2 saturation was $>95\%$ in all piglets. Heart rate and mean arterial blood pressure returned to pre-arrest levels within 1 hour (Figure 1). Heart rate was not different among HI groups. Arterial blood pressure in the hypothermia plus saline group was significantly higher than in the normothermic plus saline group at 17–19 hours of recovery and was also higher than in the normothermic plus EUK-134 group at 13–19 hours of recovery.

During HI and early recovery, rectal temperature was maintained in the normal range of 38.5–39.0°C for piglets. In the hypothermic groups, rectal temperature was rapidly decreased starting at 4 hours and reached 34°C within 25 minutes of external cooling. Thereafter, temperature was well maintained (Figure 2). Between 4.5 and 20 hours after ROSC, the mean temperature was $33.9 \pm 0.1^\circ\text{C}$ in hypothermic animals and $38.5 \pm 0.5^\circ\text{C}$ in normothermic animals. During rewarming, temperature was increased at the expected rate of approximately 1°C/h . During the remaining 10 days of recovery, rectal temperature was similar among groups.

Hypothermia did not affect the cumulative dose requirements for fentanyl (normothermic groups: $416 \pm 32 \mu\text{g/kg}$; hypothermic groups: $383 \pm 48 \mu\text{g/kg}$) or pancuronium (normothermic groups: $4.0 \pm 1.9 \text{ mg/kg}$; hypothermic groups: $3.2 \pm 2.2 \text{ mg/kg}$) from 3 hours to 24 hours after ROSC. Sham normothermic and hypothermic animals received similar cumulative doses of fentanyl ($421 \pm 67 \mu\text{g/kg}$ and $393 \pm 33 \mu\text{g/kg}$, respectively) and pancuronium ($4.2 \pm 1.5 \text{ mg/kg}$ and $3.8 \pm 0.6 \text{ mg/kg}$, respectively). The duration of mechanical ventilation after cessation of pancuronium infusion and the time until the piglets began to feed independently after extubation were not significantly different among the four HI groups, although these durations in some of the HI groups were significantly longer than those in the sham-operated group (Table 2).

Histopathology

Sham-operated, nonischemic piglets treated with hypothermia, EUK-134, or combined hypothermia and EUK-134 did not show any morphological abnormalities in putamen compared to normothermic, sham-operated piglets as determined by H&E staining (Figure 3). Hence, the four cohorts were pooled into a combined sham group for purposes of quantification. Piglets that recovered with normothermia plus saline treatment after HI had severe ischemic cytopathology in putamen, characterized by extensive ischemic changes in principal neurons with prominent nuclear pyknosis and the presence of many small cell nuclei. Very few neurons with normal morphology were evident. In contrast, piglets that recovered with hypothermia plus saline treatment, normothermia plus EUK-134 treatment, and hypothermia plus EUK-134 treatment had a significant number of viable neurons with normal morphology. However, cells with ischemic cytopathology were still evident, and the number of neurons with normal morphology was less than that in the sham-operated piglets (Figure 3).

Profile counting of viable neurons in central putamen indicated severe loss of neurons at 10 days of recovery from HI in the normothermic group treated with saline vehicle (Figure 4A). The number of viable neurons in putamen was significantly increased by treatment with hypothermia plus saline ($P < 0.001$), normothermia plus EUK-134 ($P = 0.007$), and hypothermia plus EUK-134 ($P < 0.001$). However, values in these three treatment groups remained less than that in the combined sham group ($P < 0.001$). Moreover, the number of viable neurons in the group that received combined hypothermia and EUK-134 treatment did not differ significantly from that of the groups treated with hypothermia alone ($P = 0.89$) or EUK-134 alone ($P = 0.39$). Confidence intervals for pairwise comparisons of the differences in the mean values among groups are displayed in Figure 5. The confidence interval for the differences between combined treatment and hypothermia alone was less than 20% of the mean sham value. Thus, a true difference between EUK-134 plus hypothermia versus hypothermia alone has a low likelihood of being physiologically meaningful.

In caudate nucleus, cell loss after HI in the normothermic plus saline group was less severe than that in putamen, as reported before in untreated HI groups.^{19,20} Nevertheless, neuroprotection was observed with the three treatments, although values remained less than

that in the sham group (Figure 4C). Comparisons of combined hypothermia and EUK-134 treatment with hypothermia alone ($P = 0.84$) or EUK-134 alone ($P = 0.95$) were not significant, and confidence intervals of the differences in mean values were less than 20% of the sham value (Figure 5).

In parasagittal neocortex, the number of viable neurons after HI in the normothermic plus saline group was less than that in the sham group, although the decrease was more variable than in the striatum (Figure 4D). The density of viable neurons in groups treated with hypothermia plus saline, normothermia plus EUK-134, and hypothermia plus EUK-134 after HI was not significantly different from that in the normothermic plus saline group, presumably because of the high variability in the latter group. However, the density of cortical neurons in the three treatment groups was higher on average than that in the normothermic plus saline group and not significantly different from the value in the sham group. No trend was evident for superior cortical protection with combined treatment compared to individual treatment (Figure 5).

Because our profile counting analysis indicated that injury was most profound in putamen, this region in the opposite hemisphere was reanalyzed with the use of an unbiased stereological approach in a subset of piglets. In general, the results from this analysis corroborated those found with profile counting (Figure 4B).

Neurologic deficit score

At 4 days of recovery from HI, the median neurologic deficit score was 29 in the normothermia plus saline group, 8 in the hypothermia plus saline group, 10.5 in the normothermia plus EUK-134 group, and 9 in the hypothermia plus EUK-134 group (Figure 6). The Kruskal-Wallis analysis of ranks indicated $P = 0.16$.

Discussion

The results of the present study failed to support our hypothesis that early treatment with the antioxidant EUK-134 at 30 minutes after reoxygenation from neonatal HI would have an additive neuroprotective benefit with hypothermia that was delayed by a clinically relevant duration of 4 hours. The endpoint of viable neurons was the measure of neuroprotection and was assessed using two different cell counting methods. Both methods yielded similar results. Thus, despite the limitations of profile counting-based methods, this approach is nevertheless valid. Several possibilities need to be discussed to interpret this inefficacy of EUK-134 to augment hypothermic neuroprotection.

The subpopulation of striatal neurons protected by EUK-134 may be the same subpopulation that is able to be protected by delayed hypothermia. Thus, a different subpopulation of striatal neurons that are highly vulnerable to HI might not be protected by either intervention. The operative mechanisms for hypothermic neuroprotection could be numerous, and the exact mechanisms have not been identified clearly. Because there was no detectable additive effect, our results suggest that EUK-134 and hypothermia may have a common mechanism of blocking oxidative stress, either by attenuating superoxide/hydrogen peroxide production or by increasing scavenging of these oxidants.

Another possibility is that the level of oxidative stress in a subpopulation of neurons may already induce necrosis or downstream cell death signaling that can no longer be reversed when treatment with EUK-134 is delayed by 30 minutes. We did not administer EUK-134 immediately after reoxygenation because such a short delay would not be clinically feasible. Moreover, administration of EUK-134 at 30 minutes was effective in reducing protein nitration and carbonyl formation that are normally seen at 3 hours after HI. Nevertheless,

neuronal nitric oxide synthase is already increased in the membrane fraction of striatal homogenates by 5 minutes after reoxygenation²³ and may already be producing substantial nitrative and oxidative stress by 30 minutes in a subpopulation of neurons. In addition, phosphorylation of NR1 of the N-Methyl-D-Aspartate receptor is already evident by 5 minutes after reoxygenation²³ and may lead to augmented calcium entry. Phosphorylation of NR1 at Ser897 may be important, because it has been reported that a D1 receptor antagonist²⁸ or induction of hypothermia at 5 minutes of recovery²³ reduces HI-induced phosphorylation at this protein kinase A-sensitive site and is associated with protection of piglet striatal neurons. Thus, an antioxidant may need to be administered immediately upon reoxygenation to obtain maximum efficacy and permit an additive neuroprotective effect with delayed hypothermia. Furthermore, EUK-134 is a manganese-salen-based antioxidant that was selected because of its efficacy in an adult rat stroke model³⁰ and its ability to reduce markers of oxidative stress in the current piglet model.²⁴ Nevertheless, newer generations of manganese-based catalytic antioxidants^{27,31} might be more effective than EUK-134 and provide additive protection with hypothermia. Alternatively, the efficacy of EUK-134 may have been greater if the piglets had been maintained on 100% O₂ during early recovery, which is often practiced clinically and which may augment oxidative stress. In the present study, 50% O₂ was used for resuscitation to offset the acidosis-induced shift in the oxyhemoglobin dissociation curve, and the inspired O₂ concentration was gradually reduced to 30% as the acidosis was corrected over the first 30 minutes of ROSC.

Another consideration is that hypothermia is thought to protect the brain by multiple mechanisms,³² and demonstration of statistical significance of additional protection by another therapy may be difficult. Interestingly, ventilation with 50% xenon immediately during recovery has been shown to exert a statistically significant additive neuroprotective effect with immediate induction of hypothermia after HI in piglets.⁹ Thus, there may be mechanisms of injury that are not targeted by hypothermia but that are targeted by another therapy such as xenon. However, in a model of HI induced by 30-minute hypoxia followed by 7-minute asphyxia, immediate induction of hypothermia was able to completely block loss of viable neurons in putamen.⁷ Although this duration of hypoxia was less severe than the 40-minute duration that we used, these previous results indicate that hypothermia can target the multiple mechanisms involved in piglet striatum injury when it is initiated rapidly.

Another point to consider is that the duration of hypothermia was limited to 1 day because of the logistical difficulty of maintaining paralyzed, sedated piglets on a ventilator for multiple nights in a laboratory intensive care setting. In the clinical setting, therapeutic hypothermia is usually maintained for 2–3 days. When the onset of hypothermia is delayed and the evolution of the injury process has already progressed, a longer duration of hypothermia may be required to achieve maximum benefits in preventing delayed neurodegeneration.³²

Finally, it is conceivable that hypothermia or prolonged sedation with fentanyl has adverse side effects that prevent additive neuroprotection with EUK-134. However, in sham-operated piglets treated with saline, EUK-134, hypothermia, or EUK-134 plus hypothermia, we did not see evidence of abnormal histopathology. This finding is consistent with previous work in which normothermic and hypothermic piglets were sedated with fentanyl for 24 hours.⁷ Moreover, a positive effect of delayed hypothermia was able to be detected when both normothermic- and hypothermic-treated encephalopathic newborns received sedation with opioids.⁴

With a 4-hour delay in instituting hypothermia, significant but incomplete neuroprotection was evident in striatum in the absence of EUK-134. This result is consistent with improved outcome in clinical studies in which the delay to induction of hypothermia after birth was

usually 3–6 hours.^{1,2,4} However in a different piglet model of HI involving 45 minutes of more severe hypoxia (5–6% inspired oxygen), delaying hypothermia by 3 hours after reoxygenation was no longer significantly effective in improving global histopathological scores.¹³ One possible explanation is that the therapeutic time window for hypothermia is shorter when the insult is more severe. This severity-related impotency of hypothermia would be consistent with the clinical head cooling trial, which found that hypothermia instituted within 6 hours of birth was less effective in those presenting with more severe symptoms.² Furthermore, analysis of magnetic resonance images indicated that hypothermia reduced the incidence and severity of basal ganglia and thalamic lesions to a greater extent in newborns with moderate reductions in amplitude-integrated electroencephalographic activity than in those presenting with severe reductions during the first 6 hours after birth.¹⁷ In contrast, a recent trial with robust whole-body cooling was able to demonstrate efficacy in those with severe encephalopathy despite an average delay in initiating hypothermia of 5 hours after birth and an additional 1.6 hours to achieve the target temperature.⁴

In previous work with the same model of HI in piglets, administration of EUK-134 at 30 minutes after reoxygenation increased the number of viable neurons in putamen at 4 days of recovery to 41% of that in sham-operated piglets.²⁴ In the present study, the recovery period was extended to 10 days. Treatment with the same dose of EUK-134 at 30 minutes after reoxygenation resulted in 40% of the neurons remaining viable. Consistent with previous work,^{19,20} neuronal loss was more severe in putamen than in caudate nucleus in the normothermic HI group. Despite the less severe neuronal loss, statistically significant increases in the number of viable neurons still could be detected with EUK-134, hypothermia, and combined treatment in caudate nucleus. Therefore, the benefit of these treatments occurred throughout the striatum. The magnitude of the improvement with EUK-134 treatment after 10 days of recovery is similar to what was previously found in caudate nucleus after a 4-day recovery period.²⁴ Thus, the protection afforded by EUK-134 and hypothermia appear to be sustained.

The peri-Rolandic neocortex is also selectively vulnerable to HI in term newborns; this vulnerability has been attributed to developmentally regulated increases in metabolic energy demand.³³ In piglets, the parasagittal neocortex at the coronal level of the striatum has selectively high activity of cytochrome oxidase.¹⁹ This observation is consistent with metabolic development of primary sensorimotor cortex preceding metabolic development in other areas of neocortex at this stage of development in piglets. This area of neocortex is also selectively vulnerable in piglets, and this selectivity is likely related to high metabolic activity in this region rather than a vascular watershed distribution. In the present study, neuronal viability 10 days after HI in the normothermic group was significantly reduced to about half of that in the sham animals, whereas values in the three HI posttreatment groups were not significantly different from the value in the sham group. Although this result suggests that each of the treatments benefited this vulnerable region of neocortex, the values in the three HI posttreatment groups were not significantly different from that in the normothermic HI group, in part, because of the high variability of injury in this region with the present model. This variability may be associated with the presence of electrical seizure activity. Our study cannot address this because electroencephalographic activity was not monitored. Moreover, unbiased stereology was not performed in cortex to correct for possible expansion or contraction of injured tissue. However, in this model of selective neuronal vulnerability, expansion or shrinkage of brain structures is not prominent.

Many piglets had detectable neurological deficits at 4 days of recovery from HI. The median values of the three treatment groups were similar and were below the 25th percentile of the normothermia-saline group. However, the values were not significantly different because of

the considerable variability among animals, and a larger sample size may be needed to demonstrate significant differences.

Hypoxia and asphyxia produced variable increases in blood glucose concentration that may have contributed to the variability in outcome. However, we previously found that hyperglycemia was not associated with adverse outcome; rather, decreases in glucose below 80 mg/dL after resuscitation were associated with worse neurological deficits.³⁴ Therefore, piglets received 5% dextrose infusion to maintain glucose stores and prevent hypoglycemia. Moreover, hyperglycemia has not been found to adversely affect outcome in other models of ischemic injury in immature brain as it does in mature brain.^{35,36}

In summary, treatment of newborn piglets with the antioxidant EUK-134 at 30 minutes after reoxygenation or treatment with hypothermia at 34°C for one day starting at 4 hours of recovery from HI partially protected striatal neurons. However, combining early antioxidant treatment with delayed hypothermia did not produce a statistically superior benefit, and the confidence intervals suggest that any true additional benefit would unlikely be meaningful. Other approaches will be necessary to extend the therapeutic window for induced hypothermia in newborns after HI.

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Figure 1.

Arterial O₂ saturation, heart rate, and mean arterial blood pressure at baseline, at 5, 15, 27, and 37 minutes of a 40-minute period of hypoxia, at 5 minutes of a 5-minute period of room air (RA) ventilation, during 7 minutes of asphyxia (Asph), and during the first 24 hours of recovery. After reoxygenation, piglets were treated with normothermia plus saline vehicle, normothermia plus EUK-134, hypothermia plus saline vehicle, or hypothermia plus EUK-134. Saline or EUK-134 administration started at 0.5 hours, and hypothermia started at 4 hours. Values are shown as means $\pm$ SD. Mean arterial blood pressure in the hypothermia plus saline group was significantly greater than in the normothermic plus saline group at 17–19 hours of recovery and was also greater than in normothermic plus EUK-134 group at 15 and 27 minutes of hypoxia and at 13–19 hours of recovery. The sham-operated groups were omitted for clarity, but values were similar to those attained during recovery from hypoxia and asphyxia. HI, hypoxia-ischemia.

Figure 2.

Time course of rectal temperature. Piglets were treated with saline or EUK-34 at 30 minutes of recovery from hypoxia and ischemia (HI) and then were exposed to normothermia or whole body mild hypothermia starting at 4 hours of recovery. Values are shown as means $\pm$ SD. Sham-operated groups were omitted for clarity.

Figure 3.

Examples of hematoxylin and eosin-stained sections of putamen at 10 days after sham surgery (upper panels) or hypoxia-ischemia (lower panels) in piglets treated with saline vehicle and normothermia, saline vehicle and hypothermia, EUK-134 and normothermia, or EUK-134 and hypothermia. All sham groups showed viable neurons, with large nuclei and distinct nucleoli, and smaller glial cells. Normothermia without EUK-134 treatment after hypoxia-ischemia showed profound neuronal degeneration as indicated by the neuronal shrinkage and pyknotic nuclei. With hypothermia alone, EUK-134 alone, or combined treatment, many neurons exhibited normal appearance, but shrunken cells with pyknotic nuclei were still evident. Scale bars = 20 μ m.

Figure 4.

Neuronal viability in putamen, caudate nucleus, and neocortex. The number of viable neurons at 10 days of recovery was assessed by profile counting in putamen (A), caudate nucleus (C), and in layer V of primary sensorimotor cortex (D) and is expressed as a percentage of viable neurons in the combined sham groups ($n=8$). Hypoxia-ischemia (HI) and treatment with saline vehicle plus normothermia ($n=11$); HI and saline vehicle plus hypothermia ($n=10$); HI and EUK-134 plus normothermia ($n=10$); HI and EUK-134 plus hypothermia ($n=10$). Volumetric density of viable neurons (B) was assessed in contralateral putamen by unbiased stereology in subsets of piglets in the combined sham groups ($n=7$); HI and treatment with saline vehicle plus normothermia ($n=6$); HI and saline vehicle plus hypothermia ($n=6$); HI and EUK-134 plus normothermia ($n=5$); HI and EUK-134 plus hypothermia ($n=8$). Values are means $\pm$ 95% confidence intervals; * $P < 0.05$ vs. HI normothermia and saline group; + $P < 0.05$ vs. combined sham group by analysis of variance and Holm-Sidak procedure.

Figure 5.

Mean and 95% confidence intervals of the differences between the means of pairwise comparisons among the five groups of profile neuronal counts in putamen, caudate nucleus, and neocortex. Confidence intervals were corrected for multiple comparisons by the Holm-Sidak procedure. HI, hypoxia-ischemia, NT, normothermia, EUK, EUK-134, HT, hypothermia.

Figure 6.

Box-whisker plots show 5, 25, 50, 75, and 95 percentiles of neurological deficit score (maximum deficit = 154) at 4 days of recovery from hypoxia-ischemia (HI) and treatment with normothermia plus saline, hypothermia plus saline, normothermia plus EUK-134, and hypothermia plus EUK-134. $P = 0.16$ by Kruskal-Wallis test.

Table 1

Arterial pH, blood gases, and glucose

	Baseline		Hypoxia		Room air		Asphyxia		Post-resuscitation			
			37 min		5 min		5 min		6 h	12 h	24 h	
pH												
HI+NT+saline	7.44±0.03	7.36±0.10	7.35±0.13	6.91±0.09	7.41±0.03	7.40±0.02	7.41±0.05					
HI+HT+saline	7.42±0.05	7.36±0.10	7.37±0.09	6.96±0.08	7.36±0.02 ^a	7.38±0.07	7.42±0.06					
HI+NT+EUK134	7.43±0.04	7.42±0.05	7.42±0.06	6.98±0.09	7.43±0.06 ^b	7.45±0.06 ^b	7.41±0.05					
HI+HT+EUK134	7.44±0.08	7.38±0.11	7.40±0.12	6.99±0.09	7.43±0.06 ^b	7.42±0.04	7.40±0.10					
PaO₂ (mmHg)												
HI+NT+saline	141±14	22±3	67±15	8±4	135±17	131±17	125±40					
HI+HT+saline	144±19	20±2	81±9	10±3	158±15 ^a	153±24 ^a	119±42					
HI+NT+EUK134	137±15	24±4	72±13	8±2	130±22 ^b	146±24	135±23					
HI+HT+EUK134	145±20	21±2	83±16	7±4	154±25 ^a	160±13 ^a	122±37					
PaCO₂ (mmHg)												
HI+NT+saline	37±3	38±5	38±8	93±17	37±5	37±5	35±4					
HI+HT+saline	39±4	38±5	35±4	79±10	40±5	39±8	35±6					
HI+NT+EUK134	42±3	37±5	37±5	95±15	37±6	34±5	35±6					
HI+HT+EUK134	40±4	38±4	34±5	84±11	40±7	39±6	40±9					
Glucose (mg/dL)												
HI+NT+saline	93±27	185±79	173±80	194±119	75±21	90±26	85±42					
HI+HT+saline	109±38	192±64	158±57	263±128	71±35	135±67	123±47					
HI+NT+EUK134	86±18	129±43	125±42	155±94	78±30	85±18	107±30					
HI+HT+EUK134	92±19	137±72	152±58	181±99	84±45	109±43	112±38					

Values are means ± SD in hypoxia-ischemia (HI) groups treated with normothermia (NT) and saline, hypothermia (HT) and saline, NT and EUK-134, and HT and EUK-134.

^a $P < 0.05$ from NT+saline;

^b $P < 0.05$ from HT+saline.

Table 2

Intensive care management variables

Sham	HI+NT+saline	HI+HT+saline	HI+NT+EUK134	HI+HT+EUK134
Pre-arrest body weight (kg)				
1.8(1.6,1.9)	2.3(2.1,2.7) ^a	2.2(2.1,2.6) ^a	2.3(2.1,2.7) ^a	2.3(2.1,2.6) ^a
Cumulative epinephrine dose (mg)				
	0.33(0.33,0.58)	0.33(0.00,0.33)	0.33(0.33,0.66)	0.33(0.33,0.33)
Duration of cardiopulmonary resuscitation (min)				
	1.5(1.0,2.0)	1.5(1.0,2.2)	1.5(1.0,2.5)	1.6(1.0,2.0)
Duration of mechanical ventilation after cessation of pancuronium (h)				
3.8(3.3,4.3)	5.0(4.5,5.8)	5.0(4.5,8.0)	4.5(4.0,5.5)	5.3(4.5,7.5) ^a
Time to feed independently after extubation (h)				
6(3.5,9)	16(10,23)	22(10,30) ^a	15(10,30)	24(10,30) ^a

Values are medians (25th, 75th quartiles) in sham-operated group and hypoxia-ischemia (HI) groups treated with normothermia (NT) and saline, hypothermia (HT) and saline, NT and EUK-134, and HT and EUK-134.

^a $P < 0.05$ from sham; there were no significant differences among HI groups.