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White matter apoptosis is increased by delayed hypothermia and rewarming in a neonatal piglet model of hypoxic ischemic encephalopathy

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Abstract

Therapeutic hypothermia is widely used to treat neonatal hypoxic ischemic (HI) brain injuries. However, potentially deleterious effects of delaying the induction of hypothermia and of rewarming on white matter injury remain unclear. We used a piglet model of HI to assess the effects of delayed hypothermia and rewarming on white matter apoptosis. Piglets underwent HI injury or sham surgery followed by normothermic or hypothermic recovery at 2 h. Hypothermic groups were divided into those with no rewarming, slow rewarming at 0.5°C/h, or rapid rewarming at 4°C/h. Apoptotic cells in the subcortical white matter of the motor gyrus, corpus callosum, lateral olfactory tract, and internal capsule at 29 h were identified morphologically and counted by hematoxylin & eosin staining. Cell death was verified by TUNEL assay. White matter neurons were also counted, and apoptotic cells were immunophenotyped with the oligodendrocyte marker 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase). Hypothermia, slow rewarming, and rapid rewarming increased apoptosis in the subcortical white matter relative to normothermia ($p < 0.05$). The number of white matter neurons was not lower in groups with more apoptosis after hypothermia or rapid rewarming, indicating that the apoptosis occurred among glial cells. Hypothermic piglets had more apoptosis in the lateral olfactory tract than those that were rewarmed ($p < 0.05$). The promotion of apoptosis by hypothermia and rewarming in these regions was independent of HI. In the corpus callosum, HI piglets had more apoptosis than shams after normothermia, slow rewarming, and rapid rewarming ($p < 0.05$). Many apoptotic cells were myelinating oligodendrocytes identified by CNPase positivity. Our results indicate that delaying the induction of hypothermia and rewarming are associated with white matter apoptosis in a piglet model of HI; in some regions these temperature effects are independent of HI. Vulnerable cells

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include myelinating oligodendrocytes. This study identifies a deleterious effect of therapeutic hypothermia in the developing brain.

Keywords

asphyxia; heart arrest; hypothermia; white matter; newborn; rewarming

INTRODUCTION

Neonatal hypoxic-ischemic encephalopathy (HIE) affects approximately 3 in 1000 births in the United States (Graham et al., 2008). Therapeutic hypothermia is the primary brain-focused treatment available for HIE, but clinical trials of hypothermia have shown limited success. In the Total Body Hypothermia for Neonatal Encephalopathy Trial for HIE, the level of neurologic disability at age 6–7 years remained substantial in many of the survivors who received therapeutic hypothermia (Azzopardi et al., 2014). In the National Institute of Child Health and Human Development Neonatal Research Network trial of therapeutic hypothermia in HIE, 35% of survivors who received hypothermia developed moderate to severe disabilities, and 17% developed cerebral palsy (Shankaran et al., 2012). In the United States, the costs of HIE-related cerebral palsy exceed \$1.9 billion per year, and the financial burden of HIE-related intellectual disabilities with IQ<70 exceeds \$3.4 billion per year (Graham et al., 2008; Martin et al., 2005; Centers for Disease Control and Prevention (CDC), 2004). Therefore, the limitations and potential risks of therapeutic hypothermia must be identified.

Induction of hypothermia after birth is often delayed in clinical situations (Shankaran et al., 2005), but the impact of this delay after hypoxic-ischemic (HI) injury is not well studied in HIE. In addition, adverse effects of rewarming could potentially neutralize some neuroprotective benefits and may be partially responsible for the persistent neurologic disabilities in survivors of HIE. We found previously that rewarming from hypothermia increases cortical neuron apoptosis and caspase-3 cleavage in a neonatal piglet model of HI brain injury (Wang et al., 2015). Because the white matter is also vulnerable in HIE (Martinez-Biarge et al., 2012; Tekes et al., 2015; Howlett et al., 2013), excitotoxicity (Wegleiter et al., 2014), and metabolic perturbations such as leukodystrophy and leukoencephalopathies (Francis et al., 2014), we theorized that rewarming from delayed hypothermia would promote apoptosis in white matter. Therefore, we used a piglet model of HI to test the hypotheses that (1) delaying hypothermia by 2 h after HI will increase glial apoptosis above the baseline of normothermic uninjured piglets; (2) rewarming from hypothermia after HI will further promote apoptosis; and (3) the rewarming rate will influence the severity of apoptosis in white matter.

EXPERIMENTAL PROCEDURES

In a previous study, we reported that HI in piglets followed by hypothermia induction at 2 h and subsequent rewarming increased apoptosis in the cortical layers of motor gyrus and caspase-3 cleavage in the sensorimotor cortex (Wang et al., 2015). To reduce the number of animals used in the current investigation and test the effects of HI, delayed hypothermia, and

rewarming on white matter apoptosis, we used white matter samples from the same piglets used in the previous study (Wang et al., 2015). Three sham groups received delayed hypothermia, delayed hypothermia+slow rewarming, or delayed hypothermia+rapid rewarming.

Animal preparation

The Johns Hopkins University Animal Care and Use Committee approved all procedures, and all protocols were in accord with the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. Animal care ensured comfort at all times and was in compliance with the National Institutes of Health Guidelines. Male piglets (3–5 days old, 1–2.5 kg) received HI injury or underwent sham surgery. Piglets within the 3–5 days of age variation were equally distributed among groups, resulting in a range of median ages of 3–4 days within each experimental group. Male, age-matched, naïve piglets that did not receive anesthesia, surgery, or HI served as an additional control group.

We anesthetized piglets as previously described (Wang et al., 2015) with oxygen, nitrous oxide, and isoflurane through a nose cone. We orally intubated the piglets and used mechanical ventilation to maintain normal oxygenation and normocapnea. After placement of femoral arterial and venous catheters (which takes approximately 10–15 min), the isoflurane was discontinued, 70% nitrous oxide with 30% oxygen was administered, and a bolus of 20 µg/kg fentanyl was delivered by intravenous (IV) injection followed by an infusion at 10–20 µg/kg/h. We administered IV vecuronium at 0.2 mg/kg/h to all piglets to prevent shivering during hypothermia and to administer the same anesthetic across all treatment groups. We previously determined that the anesthetic combination of nitrous oxide +fentanyl+vecuronium produces levels of cortical apoptosis that are comparable to those of naïve piglets that do not receive anesthesia (Wang et al., 2015).

Throughout the experiments, piglets received maintenance IV fluids with 5% dextrose in 0.45% saline at 10 ml/h. The piglets' mean arterial blood pressure (MAP) was maintained at 45 mmHg to ensure that the cerebral perfusion pressure exceeded the lower limit of autoregulation during anesthesia (Larson et al., 2013). Low-dose phenylephrine or dopamine was infused as necessary to meet this hemodynamic goal.

HI injury

Piglets were randomized to sham surgery or HI injury before induction of anesthesia. We have previously described our HI injury protocol.(Wang et al., 2015; Lee et al., 2011; Lee et al., 2012; Larson et al., 2013) Briefly, we reduced the inspired oxygen concentration to 10% for 45 min. Then we supplied 21% oxygen for 5 min; this brief reoxygenation period is required for successful cardiac resuscitation. Next we induced asphyxia for 7 min by clamping the endotracheal tube. Piglets were resuscitated with 50% oxygen, chest compressions, and 100 µg/kg epinephrine. Piglets that did not achieve return of spontaneous circulation (ROSC) after 3 min of chest compressions were excluded. After piglets attained ROSC, the oxygen concentration was reduced to 30% within 1–2 minutes of ROSC. The inhaled oxygen concentration remained at 30% for the remainder of the experiment. Sodium

bicarbonate was administered to correct the resultant metabolic acidosis, and calcium chloride was administered for hypocalcemia.

Temperature

Piglets that underwent HI injury or sham surgery were further randomized to receive (1) normothermia (38.5–39.5°C), (2) whole body hypothermia (34.0°C), (3) hypothermia followed by rapid rewarming (4°C/h), or (4) hypothermia followed by slow rewarming (0.5°C/h) until the goal sample size in each treatment group was reached. We previously reported findings on cortical apoptosis in piglets of the HI, normothermic sham, and naïve (unanesthetized) groups. (Wang et al., 2015) For the current examination of white matter apoptosis, we added three sham groups with hypothermia, hypothermia+slow rewarming, or hypothermia+rapid rewarming. Normothermia was maintained with heating lamps and warming blankets. In piglets destined to receive hypothermia, the cooling was started at 2 h to mimic clinical delays in initiating hypothermia. Beginning 2 h after ROSC, whole body hypothermia was induced by applying ice packs and a cooling blanket. In this model, it takes ~30 min for rectal temperature to reach 34°C; it has been shown that rectal temperature approximates the brain temperature within 0.2°C (Agnew et al., 2003). In piglets that were randomized to receive rewarming at either 4°C/h or 0.5°C/h, whole body temperature was increased beginning at 20 h of ROSC (after 18 h of hypothermia) by applying warm packs and increasing the temperature of the water circulating through the blanket to reach normothermia (38.5°C). Piglets in all treatment groups were euthanized at 29 h after ROSC or sham surgery.

Histology and cell death detection

Piglets were deeply anesthetized with 50 mg/kg pentobarbital and exsanguinated by transcardial perfusion with cold phosphate-buffered saline (PBS) and 4% paraformaldehyde for brain fixation. The brains remained *in situ* overnight and then were removed; forebrain slabs were embedded in paraffin, and 10-µm sections were cut. Coronal sections were matched by anterior-posterior level based on anatomic regions.

The sections were stained with hematoxylin and eosin (H&E). At the striatal anatomic level, investigators who were blinded to treatment group counted apoptotic profiles and neurons in the subcortical white matter of the motor cortical gyrus and apoptotic profiles in the corpus callosum, first discrete bundle of the internal capsule nearest to the cingulate gyrus and ventricle, and lateral olfactory tract (B.W. and J.K.L.) on one slide (one level) at 400× magnification (Fig. 1A). Investigators (B.W. and J.K.L.) blinded to treatment group also counted apoptotic profiles in the subcortical white matter of the motor gyrus at a posterior hippocampal level on one slide (one level) at 400× magnification (Fig. 1B). If there was a sulcus in the motor gyrus, then the subcortical white matter in the most medial gyrus in the parasagittal plane was counted. The identity of the motor gyrus was based on a stereotaxic atlas and physiologic descriptions (Salinas-Zeballos et al., 1986). The region of interest in the corpus callosum was defined by the white matter tract from the sulcus between the motor and cingulate gyri to the midline. Another investigator (L.J.M.) screened for counter reliability and accuracy. Apoptotic profiles were identified as cells with a few (<4) crescent-shaped or spherical clumps of chromatin, cytoplasmic condensation and cell shrinkage, an

apparent intact cytoplasmic membrane, and eosinophilic cytoplasm (Northington et al., 2007). We did not count apoptotic profiles that were within or adjacent to blood vessels in an effort to avoid including apoptotic endothelial and white blood cells.

To specifically detect dying cells with DNA fragmentation, we used the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Penzberg Germany) to identify nuclei that were positive for terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) in paraffin-embedded sections as previously described (Wang et al., 2015). This method does not distinguish between the different forms of cells death (Martin et al., 1998). TUNEL-positive cells were visualized with 3,3'-diaminobenzidine, and the sections were counterstained with cresyl violet. We treated positive control sections with DNase and omitted TdT from negative controls. Two investigators (B.W., J.K.L.) blinded to treatment group counted TUNEL+ profiles in the subcortical white matter of the motor gyrus at the striatal level on one slide (one level) and at the posterior hippocampal level on one slide (one level) at 400× magnification. Accuracy and counter reliability were again verified by another investigator (L.J.M.). We excluded TUNEL+ cells within or adjacent to blood vessels to avoid counting TUNEL+ endothelial cells and white blood cells.

To determine the cell type of the apoptotic cells, we immunophenotyped myelinating oligodendrocytes immunohistochemically using 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) antibody. CNPase is a specific marker for myelinating oligodendrocytes (Barrett et al., 2012). Sections were deparaffinized and rehydrated, rested in 0.4% triton/TBS, and blocked with 3% normal goat serum in 0.1% triton/TBS for 1 h. We then incubated the sections with the primary antibody mouse anti-CNPase IgG (1:50, Merck Millipore, Darmstadt, Germany) overnight at room temperature. After being rinsed in TBS, the sections were incubated with the secondary antibody goat anti-mouse IgG (1:20, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. The sections were rinsed again and then exposed to mouse peroxidase anti-peroxidase soluble complex antibody (1:100, Sigma-Aldrich, St. Louis, MO) with 2% normal goat serum for another hour. We developed the sections with 3,3'-diaminobenzidine substrate and applied a cresyl violet counterstain. Two investigators (L.J.M. and J.K.L.) screened apoptotic profiles for CNPase-positive oligodendrocytes. Criteria for identifying CNPase-positive apoptotic profiles included glial cells (<10 µm in diameter) with brown intracellular staining and apoptotic bodies stained by cresyl violet.

Immunoblotting

We examined caspase-3 cleavage by immunoblotting for procaspase-3 fragments as previously described (Wang et al., 2015). We have tested several caspase-3 antibodies in piglet tissue with a positive control of recombinant caspase-3 (Biovision) by Western blotting, and all the antibodies showed bands for cleaved caspase-3, procaspase, and non-specific immunoreactivity to proteins unrelated to caspase-3 (unpublished results). Because we could not identify an antibody suitable for immunohistochemistry in piglet tissue, we measured caspase-3 cleavage by Western blotting.

To minimize the number of animals, we sampled tissue from fresh brain slabs using micropunches from the subcortical white matter of the sensorimotor cortex from the piglets

in our prior cortical apoptosis study (Wang et al., 2015). We randomized separate piglets to sham surgery with normothermia, HI+delayed hypothermia, HI+rapid rewarming, or HI+slow rewarming. Naïve piglets that were not anesthetized and that did not undergo surgery or HI were prepared as an additional control group. At 29 h after ROSC or sham surgery, piglets were deeply anesthetized and killed by transcardial perfusion with cold PBS, and the brain was harvested.

Subcortical white matter samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fractionated proteins were electroeluted onto nitrocellulose membranes and then incubated with the primary polyclonal goat anti-caspase-3 IgG (1:1000, R&D Systems, Minneapolis, MD) and monoclonal mouse anti- β -actin IgG (1:3000, Santa Cruz Biotechnology, Dallas TX). After rinsing, we incubated the membranes with the secondary antibodies (anti-goat IgG 1:3000, Thermo Scientific; anti-mouse IgG 1:3000, General Electric Healthcare Life Sciences, Pittsburgh, PA) for 1 h at room temperature. After washing the membrane, we detected immunoreactivity by enhanced chemiluminescence (Thermo Scientific) to quantify the optical density and used ImageJ (National Institutes of Health, Bethesda, MD) to analyze the intensities of the immunoreactive bands. We used β -actin as the protein loading control and normalized the caspase-3 fragment optical density to β -actin for analysis. In some experiments, recombinant active caspase-3 (Biovision, Milipitas, CA) was loaded as a positive control. We ran protein from one piglet per group (naïve, sham, HI+hypothermia, HI+rapid rewarming, and HI+slow rewarming) on a single gel in four separate experiments.

Sample size calculations

We had no *a priori* knowledge of the variability and magnitude of change in white matter apoptosis with hypothermia and rewarming in this model. Therefore, we made a sample size estimate when four experiments in each of the HI+hypothermia and HI+rapid rewarming groups had been completed. Preliminary data of apoptotic profiles in the subcortical white matter indicated a difference in means of 66 between groups, with a within-group standard deviation (SD) of 34. To reject the null hypothesis that the number of apoptotic profiles would differ by <66 between rapidly rewarmed piglets and piglets that remained hypothermic after HI at an alpha level of 0.05 and power of 0.8, we calculated that we would need a sample size of 5 piglets per group. We selected a sample size of 6–8 piglets/group to allow for some error in our estimate of variability.

Statistical analysis

Data were analyzed with SigmaPlot (v11.0, Systat Software Inc., Chicago, IL) and Microsoft Excel, and graphs were generated with GraphPad Prism (v5.03, GraphPad Software Inc., La Jolla, CA). We present the data as means with SD for parametric data or medians with interquartile ranges (IQR) for nonparametric data. Differences were considered significant at $p < 0.05$.

Prior to hypothermia, differences in temperature at each time point were analyzed with one-way ANOVA and the post-hoc Holm-Sidak test (or with the Kruskal-Wallis analysis of ranks and the post-hoc Dunn's multiple comparisons test if tests of normality or equal

variance failed). The pH, PaCO₂, MAP, hemoglobin, hemoglobin oxygen saturation, sodium, potassium, and ionized calcium levels were compared among groups before and after HI with two-way repeated measures ANOVA by treatment group and time with the post-hoc Holm-Sidak test when the *F*-value was significant. Differences in pH, PaCO₂, MAP, and hemoglobin oxygen saturation during hypoxia and asphyxia were compared between HI-injured groups by one-way ANOVA (or the Kruskal-Wallis analysis of ranks when appropriate).

The number of apoptotic profiles by H&E and TUNEL stains and white matter neuron counts were compared between naïve and normothermic sham piglets using t-tests for parametric data and Mann Whitney rank sum tests for non-parametric data. To analyze the number of H&E apoptotic profiles, TUNEL profiles, and white matter neurons, data were transformed using a log(x+1) function to generate normally distributed data. Parametric data were then analyzed using 2-way ANOVA with the HI injury as factor 1 (sham or HI) and temperature as factor 2 (normothermia, hypothermia, slow rewarming, or rapid rewarming). Post-hoc pairwise comparisons using the Holm-Sidak method were used to evaluate the effects of temperature when data were stratified by sham vs. HI injury and also to evaluate the effects of HI when data were stratified by temperature.

Pairwise comparisons of H&E apoptotic and TUNEL profile counts among piglets that had measurements by both assays were analyzed with Spearman correlations. The Mann Whitney rank sums test was used to compare apoptotic and TUNEL profile counts between piglets that did and did not receive phenylephrine. Western blot densities of the caspase-3 fragment normalized to β -actin were compared by Friedman two-way analysis of ranks in which the four independent gels were blocked as a between-subject factor.

RESULTS

Mortality

Thirty-seven piglets received sham surgery, and 49 piglets underwent HI injury. Eleven naïve piglets were prepared as additional controls. Two piglets could not be resuscitated after HI, resulting in a 95% resuscitation rate. Five HI+rapid rewarming, one sham +normothermia, and two sham+slow rewarming piglets died from refractory hypotension during anesthesia. Thus, 87 piglets were used in the final analysis.

Physiology

We previously reported the temperature, pH, PaCO₂, MAP, hemoglobin, and sodium levels of the normothermic sham, HI+normothermia, HI+hypothermia, HI+slow rewarming, and HI+rapid rewarming groups.(Wang et al., 2015) Table 1 lists these values for the sham piglets that received hypothermia, slow rewarming, or rapid rewarming. During the HI protocol, the mean oxyhemoglobin saturations during hypoxia were 25% (SD: 7) in HI +normothermia (n=6), 30% (SD: 9) in HI+hypothermia (n=8), 29% (SD: 7) in HI+slow rewarming (n=8), and 29% (SD: 6) in HI+rapid rewarming (n=8) groups. During asphyxia, the oxyhemoglobin saturations decreased to 7% (SD: 11) in HI+normothermia, 7% (SD: 3) in HI+hypothermia, 4% (SD: 3) in HI+slow rewarming, and 4% (SD: 3) in HI+rapid

rewarming piglets. All piglets had severe acidosis and hypercapnea during asphyxia, but levels were restored to normal within 1 to 3 h after ROSC. The mean oxyhemoglobin saturations in all groups returned to 97% or higher after resuscitation and remained above 97% for the rest of the experiment. Pre-hypothermia rectal temperatures were similar among HI and sham groups ($p>0.05$). Piglets had core temperatures of approximately 34°C during hypothermia and were successfully rewarmed to normothermia without exceeding the goal temperature.

HI significantly affected pH ($p<0.001$ by two-way ANOVA) with HI piglets having lower pH than shams. With longer durations of anesthesia, the piglets became more acidotic ($p<0.001$). (Wang et al., 2015) In post-hoc comparisons, sham piglets that received hypothermia, slow rewarming, or fast rewarming had higher pH than all of the HI groups ($p<0.05$ for all comparisons) when controlling for time of anesthesia. Normothermic shams had lower pH than the three other sham groups ($p<0.05$ for all comparisons). HI and duration under anesthesia also affected PaCO₂ levels ($p<0.001$ for both HI and time by two-way ANOVA). HI piglets that received normothermia, slow rewarming, or fast rewarming had higher PaCO₂ levels than did shams that received slow or fast rewarming ($p<0.05$ for all comparisons) when controlling for time. PaCO₂ rose overall as time progressed.

HI had a significant effect on the MAP ($p=0.018$), and there was an interaction between the effects of time and HI on MAP as the recovery period progressed ($p=0.009$ by two-way ANOVA). At ROSC 1 h, HI piglets that received hypothermia, slow rewarming, or rapid rewarming, and sham piglets that received rapid rewarming had lower MAP than normothermic shams ($p<0.05$ for all comparisons). MAP did not statistically differ between the treatment groups after 1 h of ROSC.

Hemoglobin levels did not statistically differ by HI treatment ($p=0.224$), although they did vary by time ($p<0.001$ by two-way ANOVA). At ROSC 20 h, normothermic shams had lower hemoglobin levels (7.8 ± 2.1 g/dL) than did HI+slow rewarming (10.9 ± 2.9 g/dL) and HI+fast rewarming groups (10.8 ± 2.1 g/dL; $p<0.05$ for all comparisons). Hemoglobin levels were similar between groups at all other time points. Two-way ANOVA revealed differences in sodium levels by treatment ($p=0.030$) and time ($p<0.001$). HI+slow rewarming piglets had higher sodium levels than HI+normothermia piglets ($p<0.05$) at ROSC 3 h. Sodium levels were similar between groups at all other time points. Potassium levels were similar between groups at each time point (data not shown). Sham hypothermia piglets had higher ionized calcium levels (1.28 ± 0.08 mmol/L) than HI+normothermia (0.98 ± 0.13 mmol/L; $p=0.034$) and HI+slow rewarming (1.06 ± 0.15 mmol/L; $p=0.014$) groups at ROSC 28 h, but we found no statistical differences in ionized calcium levels between treatment groups at any other time point (data not shown).

Anesthetic regimen had minimal effect on apoptosis

We first compared the degree of apoptosis in the white matter between normothermic sham piglets and naïve unanesthetized piglets to determine whether the anesthetic increased apoptosis above the normal developmental baseline for piglets 3–5 days old. In the subcortical white matter by H&E staining, the number of apoptotic profiles was similar between normothermic sham ($n=8$) and naïve ($n=7$) piglets at striatal ($p=0.062$; Fig. 2A)

and hippocampal ($p=0.960$; Fig. 2B) anatomic levels. TUNEL+ profile counts in the subcortical white matter were also similar between normothermic sham ($n=8$) and naïve ($n=7$) piglets at the striatal level ($p=0.955$; Fig. 2C) and between normothermic sham ($n=5$) and naïve ($n=6$) piglets at the hippocampal level ($p = 0.792$; Fig. 2D). In addition, similar H&E apoptotic profile counts were observed between normothermic sham ($n=8$) and naïve ($n=7$) groups in the corpus callosum ($p=0.397$), internal capsule ($p=0.536$), and lateral olfactory tract ($p=0.536$). Based on the comparable apoptotic and TUNEL+ profile counts between normothermic sham and naïve unanesthetized piglets, the anesthetic regimen had minimal effect on white matter apoptosis.

One naïve piglet did not have TUNEL staining because an appropriate hippocampal anatomic level was not available. Three normothermic shams did not have TUNEL staining because of damage to the mounted tissue in one piglet, inconsistent TUNEL stain in the second piglet, and unavailability of an appropriate anatomic level in the third piglet.

Delayed hypothermia and rewarming increased white matter apoptosis

Representative images of morphologically normal and apoptotic glial cells in the subcortical white matter are shown in Figure 3. Normothermic sham piglets had normal oligodendrocytes with scant apoptosis in white matter (Figs. 2 and 3A). HI piglets that received hypothermia with rewarming manifested apoptotic profiles by H&E and TUNEL staining (Figs. 3B and C). Apoptotic cells were immunophenotyped as CNPase-positive (Fig. 3D), thereby indicating that much of the apoptosis occurred among myelinating oligodendrocytes. HI piglets that remained normothermic had normal glial morphology and relatively infrequent apoptosis in the subcortical white matter (Figs. 3E and F).

At the striatal level with H&E stain, both HI injury ($p=0.034$) and temperature ($p<0.001$) independently affected subcortical white matter apoptosis; there was no interaction between HI and temperature on apoptosis ($p=0.054$). When data were stratified by HI condition in post-hoc comparisons, normothermic HI piglets ($n=6$) had less apoptosis than did hypothermic ($p=0.030$; $n=8$), slowly rewarmed ($p=0.015$; $n=8$), and rapidly rewarmed ($p=0.014$; $n=8$; figure 4A) HI piglets. Among sham piglets, hypothermia ($p<0.001$; $n=7$), slow rewarming ($p<0.001$; $n=7$), and rapid rewarming ($p<0.001$; $n=8$) increased apoptosis above that in normothermic shams ($n=8$). In addition, hypothermic sham piglets had more apoptosis than rapidly rewarmed shams ($p=0.021$). When data were stratified by temperature, HI increased apoptosis above that observed in shams after rapid rewarming ($p=0.034$). The slow and rapid rewarming groups had comparable apoptotic counts ($p=0.967$ for HI and $p=0.290$ for sham piglets). Absolute apoptotic profile counts prior to logarithmic transformation are presented in Table 2.

We used TUNEL stain to corroborate cell death as evidenced by DNA fragmentation. At the striatal level, H&E apoptotic profile and TUNEL+ profile counts correlated well among piglets that had cell counts by both histologic staining methods ($r=0.843$; $p<0.0001$; $n=62$). Temperature ($p<0.001$), but not HI ($p=0.539$), affected the number of TUNEL+ profiles with an interaction between the two factors ($p=0.024$). Post-hoc comparisons with data stratified by injury showed that among the shams, hypothermia ($p < 0.001$; $n=7$), slow rewarming ($p<0.001$; $n=7$), and rapid rewarming ($p<0.001$; $n=8$) increased TUNEL+ profile counts

above that in normothermia ($n=8$; figure 4B). Moreover, hypothermic shams had more TUNEL+ profiles than rapidly rewarmed shams ($p=0.017$). After HI, hypothermia ($n=7$) produced more TUNEL+ profiles than did normothermia ($n=6$; $p=0.037$). Slowly rewarmed ($n=7$) and rapidly rewarmed ($n=5$) HI piglets had comparable TUNEL+ counts to HI normothermic and hypothermic piglets, and rewarming rate did not affect apoptosis after HI ($p>0.05$ for all comparisons). Cell counts prior to logarithmic transformation are presented in Table 2. TUNEL profiles could not be quantified in one HI+hypothermia piglet, two HI+rapid rewarming piglets, and two HI+slow rewarming piglets because of tissue damage on the slide.

At the hippocampal level with H&E staining, temperature ($p=0.002$), but not HI ($p=0.438$), affected apoptosis in the subcortical white matter. Temperature and HI had no interactive effect on apoptosis ($p=0.456$). Among sham piglets, both hypothermia ($p=0.040$; $n=7$) and slow rewarming ($p=0.002$; $n=7$) increased apoptosis above that observed during normothermia ($n=8$; figure 4C). Temperature did not affect apoptosis in HI piglets ($p>0.05$ for all comparisons).

Although classification of cells as apoptotic by H&E and TUNEL staining correlated among piglets in which both stains were used ($r=0.735$; $p<0.0001$; $n=39$), the TUNEL staining did not show an effect of HI ($p=0.673$) or temperature ($p=0.060$) on TUNEL profile counts at the hippocampal level (figure 4D). The TUNEL assay could not be completed in all piglets because tissue at the appropriate hippocampal anatomic level was not available in one naïve piglet, one normothermic sham piglet, two HI+normothermia piglets, one HI+hypothermia piglet, and one HI+slow rewarming piglet. The TUNEL stain was inconsistent throughout the tissue in one normothermic sham and one HI+hypothermia piglet. The mounted tissue was damaged for one piglet in each of the normothermic sham, sham+slow rewarming, HI+normothermia, and HI+slow rewarming groups.

To assess the possible contribution of white matter neurons in apoptosis, we counted neurons in the subcortical white matter of the motor gyrus at the striatal level. Figure 5A shows representative images of white matter neurons by H&E stain. These cells were very large compared to nearby non-neuronal cells and were easily distinguishable. White matter neuron counts were similar between normothermic sham ($n=8$) and naïve unanesthetized piglets ($p=0.664$; $n=7$), thereby indicating minimal effect from the anesthetic. HI ($p=0.023$), but not temperature ($p=0.576$), affected the number of white matter neurons, and these factors did not have an interactive effect ($p=0.272$). When data were stratified by injury, temperature did not affect neuron counts in HI or sham piglets ($p>0.05$ for all comparisons). Comparisons for data stratified by temperature showed that after slow rewarming, shams ($n=7$) had more white matter neurons than HI piglets ($p=0.029$; $n=8$; figure 5B). Neuron counts were similar between sham or HI piglets that received normothermia, hypothermia, or rapid rewarming ($p>0.05$ for all comparisons). These findings suggest that the white matter apoptosis observed among HI and sham piglets that received normothermia, hypothermia, or rapid rewarming occurred among glial cells. However, the decrease in white matter neurons among slowly rewarmed HI piglets suggests that neurons in addition to glia may have been involved. Absolute neuron counts are presented in Table 2.

We also counted apoptotic profiles by H&E stain in the corpus callosum, lateral olfactory tract, and superior (first bundle) internal capsule at the anterior striatal level to determine the effects of delayed hypothermia and rewarming in other white matter regions. HI ($p<0.001$), but not temperature ($p=0.355$), affected apoptosis in the corpus callosum. No interaction was observed between HI and temperature on apoptosis ($p=0.134$). When data were stratified by temperature, HI piglets had more apoptosis than shams during normothermia ($p<0.001$), slow rewarming ($p=0.005$), and rapid rewarming ($p=0.002$; figure 6A).

In the lateral olfactory tract, both HI ($p=0.014$) and temperature ($p=0.005$) independently affected apoptosis, but HI and temperature did not interact in their effects on apoptosis ($p=0.954$). When controlling for HI injury, hypothermic piglets had more apoptosis than did normothermic ($p=0.018$), slowly rewarmed ($p=0.012$), and rapidly rewarmed ($p=0.023$; figure 6B) piglets. No significant post-hoc pairwise relationships were identified when data were stratified by HI injury or temperature ($p>0.05$ for all comparisons). Neither HI injury ($p=0.591$) nor temperature ($p=0.081$) affected apoptosis in the internal capsule (figure 6C). Absolute apoptotic profile counts for these regions are presented in Table 3.

HI and rewarming did not affect levels of cleaved caspase-3

We previously reported greater cortical cleaved caspase-3 by western blotting in piglets that received HI+rapid rewarming compared to those that received HI+slow rewarming and HI +hypothermia.(Wang et al., 2015) In the same piglets, we found that western blots for cleaved caspase-3 in the subcortical white matter of the sensorimotor cortex were similar among naïve ($n=4$), normothermic sham ($n=4$), HI+hypothermia ($n=4$), HI+rapid rewarming ($n=4$), and HI+slow rewarming groups ($n=4$; $p=0.287$; figure 7).

Effects of phenylephrine/dopamine

One piglet (sham+slow rewarming) received dopamine. Six piglets received phenylephrine, including one HI+rapid rewarming, one HI+hypothermia, two HI+slow rewarming, and two HI+normothermia piglets. At the striatal level, the number of apoptotic profiles in the subcortical white matter by H&E staining did not differ between piglets that received phenylephrine (median: 17; IQR: 8,24; $n=6$) and those that did not (median: 8; 3,19; $n=39$; $p=0.204$). The number of TUNEL profiles was also similar between piglets that received phenylephrine (16; 11, 19; $n=5$) and those that did not (8; 4, 19; $n=35$; $p=0.234$). At the hippocampal level, the number of H&E apoptotic profiles in the subcortical white matter among piglets that received phenylephrine (25; 10,33; $n=5$) was similar to that of piglets that did not receive phenylephrine (12; 4, 20; $n=32$; $p=0.125$). The two piglets that received phenylephrine and had TUNEL counts at the hippocampal level had a median profile number of 27, and 18 piglets with TUNEL counts and that did not receive phenylephrine had a median profile number of 12 (IQR: 9, 17).

DISCUSSION

This study demonstrates several new findings relevant to the treatment of neonatal HIE. In a piglet model of HI, we first determined that exposure to fentanyl+nitrous oxide+vecuronium for 29 h did not increase apoptosis above that of normal developmental programmed cell

death in the subcortical white matter, corpus callosum, lateral olfactory tract, and internal capsule. Second, HI increased apoptosis in the white matter, but temperature management also independently affected apoptosis. Third, hypothermia with a delayed onset of 2 h after HI did not protect against apoptotic glial cell death. Fourth, rewarming from hypothermia, whether slow or rapid, was associated with white matter apoptosis that was significantly greater than that in normothermic piglets. The number of white matter neurons did not decrease despite greater apoptosis in normothermic, hypothermic, and rapidly rewarmed groups, thereby indicating that the apoptosis occurred primarily in glia. The decreased neuronal count in slowly rewarmed HI piglets suggests that some apoptosis may have involved neurons in addition to glia at this rewarming rate. Nonetheless, immunophenotyping with CNPase revealed that many of the apoptotic cells were myelinating oligodendrocytes. Therefore, we conclude that delayed hypothermia does not suppress glial apoptosis after HI and that rewarming permits the continuation of apoptosis in the white matter. The promotion of apoptosis by hypothermia and rewarming is independent of HI injury in some regions, including the subcortical white matter and lateral olfactory tract. The at-risk cells include myelinating oligodendrocytes. Our study identifies an untoward effect of therapeutic hypothermia in the neonatal brain.

Because of rising concerns regarding anesthesia-induced neurotoxicity (Hays and Deshpande, 2011), we evaluated the effects of anesthesia on the white matter. In a previous study, we tested different anesthetic regimens and quantified cortical apoptosis in neonatal piglets. The combination of fentanyl+nitrous oxide+vecuronium did not increase cortical neuron apoptosis above that observed in naïve unanesthetized piglets (Wang et al., 2015). Here, we extend these observations by showing that sham piglets that received fentanyl+nitrous oxide+vecuronium for 29 h with normothermia had white matter apoptotic profile counts similar to those of naïve unanesthetized piglets. Administering isoflurane for 5 h is associated with oligodendrocyte apoptosis in non-human primates (Brambrink et al., 2012). In our study, isoflurane was delivered to the piglets for approximately 10–15 min during femoral catheter placement, after which it was discontinued. Nonetheless, our findings indicate that our anesthetic regimen did not increase white matter apoptosis above developmental programmed cell death.

We designed our experimental protocols based on clinical situations. Our piglet model of HI uses a whole body ischemic-asphyxic insult, which is similar to the whole body ischemia experienced during birth injuries that cause HIE. The brain regions that are selectively vulnerable to HI in neonatal piglets correspond to those in human term newborns (Martin et al., 1997). We delayed the onset of hypothermia to mimic the clinical delay in inducing therapeutic hypothermia (Shankaran et al., 2005). Although the overnight hypothermia in our piglet experiments is shorter than the 72 h of hypothermia used clinically (Shankaran et al., 2005), it is considerably longer than the 3–8 h used in many rodent studies (Fang et al., 2013; Liu et al., 2012). The rewarming rate of 0.5°C/h is the clinical rate that neonatologists use to treat HIE (Shankaran et al., 2005). We also selected a very fast rewarming rate of 4°C/h as a comparison because we reasoned that rapid rewarming would affect cell death more than slow rewarming.

We hypothesized that delaying the induction of hypothermia by 2 h after HI would affect glial cell death in part based on the finding that near-term sheep express high levels of glutamate receptors in white matter oligodendrocytes (Furuta and Martin, 1999). High glutamate receptor levels could possibly put these cells at risk for excitotoxicity, which causes fulminate cell death along an apoptosis-necrosis continuum in the developing forebrain (Portera-Cailliau et al., 1997). Indeed, apoptosis in the subcortical white matter and lateral olfactory tract of piglets that received delayed hypothermia was elevated compared with that observed in normothermic piglets. The promotion of apoptosis by delayed hypothermia occurred in both sham and HI piglets and was therefore independent of HI injury. Hypothermia has been considered neuroprotective based on evidence that it attenuates apoptosis (Xiong et al., 2011) and suppresses the inflammatory response (Fukui et al., 2006) in neonatal rodent models of HI. However, our data in neonatal HI piglets show an association between delayed hypothermia and an increase in white matter apoptosis as compared with that in normothermic piglets, including apoptosis of myelinating oligodendrocytes. It is possible that waiting to induce hypothermia after HI permits the activation and subsequent execution of apoptotic pathways in the white matter. The promotion of apoptosis by delayed hypothermia in sham piglets, however, suggests that hypothermia itself can activate apoptotic pathways even in the absence of HI. Although a pro-apoptotic interaction between hypothermia and the anesthetic cannot be ruled out, further investigations on potential white matter toxicity from delayed hypothermia is certainly warranted. Because we tested only one time duration in delaying hypothermia, we cannot determine whether a greater delay would cause more glial apoptosis or whether immediate hypothermia would prevent apoptosis altogether. Hypothermia initiated 90 min after asphyxia prevented oligodendrocyte cell death in preterm lambs with HI (Barrett et al., 2012), whereas delaying hypothermia by 12 h after HI resulted in cerebral hemispheric loss in neonatal rats (Sabir et al., 2012). Our 2-h delay is shorter than that in clinical trials which average a 5-h delay (Shankaran et al., 2005). Additional studies are needed to clarify the risks of delaying hypothermia in neonatal HIE.

Piglets that received delayed hypothermia followed by rewarming, whether slow or rapid, had significantly more apoptosis in the subcortical white matter than did normothermic piglets. The promotion of apoptosis by rewarming in the subcortical white matter was independent of HI, and rewarming rate did not affect the degree of apoptosis. In the corpus callosum, HI-injured piglets had more apoptosis than sham piglets that received slow or rapid rewarming. Altogether, these findings suggest that delayed hypothermia and rewarming, whether slow or rapid, can promote apoptosis in the HI-injured developing brain. It is difficult to discern the differential contributions of delayed hypothermia and rewarming in promoting apoptosis, but both must be considered together because rewarming is a mandatory component of therapeutic hypothermia in clinical practice. We are unsure of the molecular mechanisms related to the hypothermia-rewarming induction of white matter apoptosis, though possibilities include sumoylation (Wang et al., 2012) or heat shock (Cullen and Sarge, 1997; Leak, 2014). In addition, rewarming might promote oxidative stress, proinflammatory cytokine shifts with complement activation, (Bisschops et al., 2011) and endoplasmic reticulum stress (Kumar et al., 2003; Hayashi et al., 2005) that culminate in apoptosis. We did not immunophenotype the apoptotic cells to determine the stage of

oligodendrocyte maturation. The presence of CNPase-positive apoptotic cells does, however, indicate that the apoptosis involved myelinating oligodendrocytes.

Our assessment of apoptosis was based on morphology discerned by H&E and TUNEL staining. Our criteria have been described before (Martin et al., 1998). Results from the two different methods correlated in two different areas of brain. However, Western blots for cleaved caspase-3 yielded results that were similar among naïve, normothermic sham, HI +hypothermia, HI+rapid rewarming, and HI+slow rewarming groups. We believe that the low sensitivity of the western blots in detecting glial apoptosis is due to limitations of using a crude homogenate assay, which lacks the cellular resolution of histologic observation and precise cell counting, and the magnitude of the pathology (table 2). The Western blot samples included not just glial proteins, but also neuronal proteins and proteins from blood-borne and vascular cells. We excluded apoptotic blood cells and endothelial cells in our histologic counts, but we did not quantify these cells and cannot make comparisons across treatment groups. While it would be ideal to test for cleaved caspase-3 using immunohistochemistry, we could not identify a suitable antibody for immunohistochemistry in piglet tissue. We tested multiple caspase-3 antibodies in piglet tissue and a recombinant caspase-3 positive control (Biovision) by Western blotting. All antibodies that we tested showed multiple bands including bands for caspase-3, procaspase, and non-specific immunoreactivity for proteins unrelated to caspase-3 (unpublished results). These antibodies are not suitable for immunohistochemistry in piglets. In addition, our sample size was small. We previously used Western blotting to show that cleaved caspase-3 was greater in the sensorimotor cortex of rapidly rewarmed HI piglets than in that of piglets that received slow rewarming or sustained hypothermia (Wang et al., 2015). We used white matter samples from the same piglets in equivalent sample sizes in this study.

Our findings that delayed hypothermia and rewarming are associated with white matter apoptosis combined with our prior report that rewarming promotes cortical apoptosis after HI (Wang et al., 2015) force us to consider the risk-benefit ratio of therapeutic hypothermia. In our piglet model, recovery from HI with normothermia results in cortical laminar necrosis (Lee et al., 2012) and putamenal necrosis (Mueller-Burke et al., 2008). Hypothermia decreases the striatal neuronal necrosis observed after HI (Agnew et al., 2003). Clinical studies indicate that therapeutic hypothermia decreases the risk of death in neonates with HIE, (Shankaran et al., 2012) although the risk of persistent neurologic disabilities remains high despite treatment with hypothermia (Shankaran et al., 2012; Azzopardi et al., 2014). Therefore, while therapeutic hypothermia confers some neurologic protection after hypoxic brain injury, this protection is incomplete. Further investigations into the risks of therapeutic hypothermia with rewarming, including the effect of delaying the induction of hypothermia, along with potential adjuvant therapies are urgently needed.

Some piglets developed an acidosis with the 29 h of continuous anesthesia, including piglets recovering from HI. Lactic acidosis would be expected after the whole body hypoxic-asphyxic cardiac arrest and was incompletely treated by sodium bicarbonate. Mild respiratory acidosis occurred in some rewarmed piglets with mild hypercarbia. This was partially due to an increase in CO₂ production with rewarming while the mechanical ventilation remained constant. Continuous anesthesia with nitrous oxide for this duration is

not used clinically and it is not well studied. It is possible that some piglets had lactate accumulation from anesthesia. Acidosis can contribute to apoptosis in astrocytes.(Giffard and Swanson, 2005) However, sham animals that exhibited more apoptosis during hypothermia or rewarming had higher pH levels than normothermic shams with less apoptosis, which suggests that acidosis did not increase apoptosis and that the observed apoptosis was related to temperature manipulation.

Our study had several limitations. The sample sizes were small but sufficient based on power calculations. Although delayed hypothermia and rewarming were associated with apoptosis, the observed effect is likely to be small relative to the total number of glia. Because we examined only one time point at 29 h after HI, we cannot determine whether more apoptosis will emerge and eventually affect the number of viable glia after longer recovery with a particular treatment, or whether the pace of cell death will subside. It is also possible that oligodendrocyte replication could reconstitute the lost oligodendrocytes. We also do not know whether the observed acute oligodendrocyte apoptosis would have any long-term neurologic consequences. Nonetheless, in clinical studies of HIE, MRI evidence of white matter injury has been observed 2 weeks after therapeutic hypothermia (Howlett et al., 2013; Tekes et al., 2015), and white matter injury correlates with greater neurodevelopmental impairments at 2 years of age (Martinez-Biarge et al., 2012). Prolonging hypothermia beyond 1 day may suppress apoptosis, as has been reported in older pigs (Suh et al., 2014). The piglets remained under anesthesia for the duration of the experiment to prevent shivering and ensure comfort. Although prolonged anesthesia (29 h) with nitrous oxide is not used clinically, the similarity in apoptosis observed between normothermic sham and naïve unanesthetized piglets and the similarity in number of white matter neurons indicates that the anesthetic did not induce apoptosis of oligodendrocytes or neurons in white matter. Finally, we examined only male piglets.

CONCLUSIONS

Identifying the potential risks of therapeutic hypothermia, including the effects of delaying the induction of hypothermia and rewarming, is critical to improving long-term neurologic outcomes. Our data suggest that delayed hypothermia and rewarming are associated with apoptosis in the subcortical white matter and lateral olfactory tract of the developing brain. The apoptotic effects of delayed hypothermia and rewarming are independent of HI injury in some white matter regions. The rate of rewarming did not influence apoptosis severity. These findings have critical implications for neonatal clinical therapeutic hypothermia protocols for HIE.

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REFERENCES

- Agnew DM, Koehler RC, Guerguerian AM, Shaffner DH, Traystman RJ, Martin LJ, Ichord RN. Hypothermia for 24 hours after asphyxic cardiac arrest in piglets provides striatal neuroprotection that is sustained 10 days after rewarming. *Pediatr Res*. 2003; 54:253–262. [PubMed: 12736390]
- Azzopardi D, Strohm B, Marlow N, Brocklehurst P, Deierl A, Eddama O, Goodwin J, Halliday HL, Juszczak E, Kapellou O, Levene M, Linsell L, Omar O, Thoresen M, Tusor N, Whitelaw A, Edwards AD, TOBY Study Group. Effects of hypothermia for perinatal asphyxia on childhood outcomes. *N Engl J Med*. 2014; 371:140–149. [PubMed: 25006720]
- Barrett RD, Bennet L, Naylor A, George SA, Dean JM, Gunn AJ. Effect of cerebral hypothermia and asphyxia on the subventricular zone and white matter tracts in preterm fetal sheep. *Brain Res*. 2012; 1469:35–42. [PubMed: 22765912]
- Bisschops LL, Hoedemaekers CW, Mollnes TE, van der Hoeven JG. Rewarming after hypothermia after cardiac arrest shifts the inflammatory balance. *Crit Care Med*. 2011
- Brambrink AM, Back SA, Riddle A, Gong X, Moravec MD, Dissen GA, Creeley CE, Dikranian KT, Olney JW. Isoflurane-induced apoptosis of oligodendrocytes in the neonatal primate brain. *Ann Neurol*. 2012; 72:525–535. [PubMed: 23109147]
- Centers for Disease Control and Prevention (CDC). Economic costs associated with mental retardation, cerebral palsy, hearing loss, and vision impairment--United States, 2003. *MMWR Morb Mortal Wkly Rep*. 2004; 53:57–59. [PubMed: 14749614]
- Cullen KE, Sarge KD. Characterization of hypothermia-induced cellular stress response in mouse tissues. *J Biol Chem*. 1997; 272:1742–1746. [PubMed: 8999855]
- Fang AY, Gonzalez FF, Sheldon RA, Ferriero DM. Effects of combination therapy using hypothermia and erythropoietin in a rat model of neonatal hypoxia-ischemia. *Pediatr Res*. 2013; 73:12–17. [PubMed: 23085817]
- Francis JS, Markov V, Leone P. Dietary triheptanoin rescues oligodendrocyte loss, dysmyelination and motor function in the nur7 mouse model of Canavan disease. *J Inherit Metab Dis*. 2014; 37:369–381. [PubMed: 24288037]
- Fukui O, Kinugasa Y, Fukuda A, Fukuda H, Tskitishvili E, Hayashi S, Song M, Kanagawa T, Hosono T, Shimoya K, Murata Y. Post-ischemic hypothermia reduced IL-18 expression and suppressed microglial activation in the immature brain. *Brain Res*. 2006; 1121:35–45. [PubMed: 17010950]
- Furuta A, Martin LJ. Laminar segregation of the cortical plate during corticogenesis is accompanied by changes in glutamate receptor expression. *J Neurobiol*. 1999; 39:67–80. [PubMed: 10213454]
- Giffard RG, Swanson RA. Ischemia-induced programmed cell death in astrocytes. *Glia*. 2005; 50:299–306. [PubMed: 15846803]
- Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. *Am J Obstet Gynecol*. 2008; 199:587–595. [PubMed: 19084096]
- Hayashi T, Saito A, Okuno S, Ferrand-Drake M, Dodd RL, Chan PH. Damage to the endoplasmic reticulum and activation of apoptotic machinery by oxidative stress in ischemic neurons. *J Cereb Blood Flow Metab*. 2005; 25:41–53. [PubMed: 15678111]
- Hays SR, Deshpande JK. Newly postulated neurodevelopmental risks of pediatric anesthesia. *Curr Neurol Neurosci Rep*. 2011; 11:205–210. [PubMed: 21222179]
- Howlett JA, Northington FJ, Gilmore MM, Tekes A, Huisman TAGM, Parkinson C, Chung SE, Jennings JM, Jamrogowicz JL, Larson AC, Lehmann CU, Jackson E, Brady KM, Koehler RC, Lee JK. Cerebrovascular autoregulation and neurologic injury in neonatal hypoxic-ischemic encephalopathy. *Pediatr Res*. 2013; 74:525–535. [PubMed: 23942555]
- Kumar R, Krause GS, Yoshida H, Mori K, DeGracia DJ. Dysfunction of the unfolded protein response during global brain ischemia and reperfusion. *J Cereb Blood Flow Metab*. 2003; 23:462–471. [PubMed: 12679723]
- Larson AC, Jamrogowicz JL, Kulikowicz E, Wang B, Yang ZJ, Shaffner DH, Koehler RC, Lee JK. Cerebrovascular autoregulation after rewarming from hypothermia in a neonatal swine model of asphyxic brain injury. *J Appl Physiol* (1985). 2013; 115:1433–1442. [PubMed: 24009008]

- Leak RK. Heat shock proteins in neurodegenerative disorders and aging. *J Cell Commun Signal*. 2014; 8:293–310. [PubMed: 25208934]
- Lee JK, Brady KM, Mytar JO, Kibler KK, Carter EL, Hirsch KG, Hogue CW, Easley RB, Jordan LC, Smielewski P, Czosnyka M, Shaffner DH, Koehler RC. Cerebral blood flow and cerebrovascular autoregulation in a swine model of pediatric cardiac arrest and hypothermia. *Crit Care Med*. 2011; 39:2337–2345. [PubMed: 21705904]
- Lee JK, Yang ZJ, Wang B, Larson AC, Jamrogowicz JL, Kulikowicz E, Kibler KK, Mytar JO, Carter EL, Burman HT, Brady KM, Smielewski P, Czosnyka M, Koehler RC, Shaffner DH. Noninvasive autoregulation monitoring in a Swine model of pediatric cardiac arrest. *Anesth Analg*. 2012; 114:825–836. [PubMed: 22314692]
- Liu Y, Shangguan Y, Barks JD, Silverstein FS. Bumetanide augments the neuroprotective efficacy of phenobarbital plus hypothermia in a neonatal hypoxia-ischemia model. *Pediatr Res*. 2012; 71:559–565. [PubMed: 22398701]
- Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Munson ML. Births: final data for 2003. *Natl Vital Stat Rep*. 2005; 54:1–116.
- Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. *Brain Res Bull*. 1998; 46:281–309. [PubMed: 9671259]
- Martin LJ, Brambrink A, Koehler RC, Traystman RJ. Primary sensory and forebrain motor systems in the newborn brain are preferentially damaged by hypoxia-ischemia. *J Comp Neurol*. 1997; 377:262–285. [PubMed: 8986885]
- Martinez-Biarge M, Bregant T, Wusthoff CJ, Chew AT, Diez-Sebastian J, Rutherford MA, Cowan FM. White matter and cortical injury in hypoxic-ischemic encephalopathy: antecedent factors and 2-year outcome. *J Pediatr*. 2012; 161:799–807. [PubMed: 22682614]
- Mueller-Burke D, Koehler RC, Martin LJ. Rapid NMDA receptor phosphorylation and oxidative stress precede striatal neurodegeneration after hypoxic ischemia in newborn piglets and are attenuated with hypothermia. *Int J Dev Neurosci*. 2008; 26:67–76. [PubMed: 17950559]
- Northington FJ, Zelaya ME, O’Riordan DP, Blomgren K, Flock DL, Hagberg H, Ferriero DM, Martin LJ. Failure to complete apoptosis following neonatal hypoxia-ischemia manifests as “continuum” phenotype of cell death and occurs with multiple manifestations of mitochondrial dysfunction in rodent forebrain. *Neuroscience*. 2007; 149:822–833. [PubMed: 17961929]
- Portera-Cailliau C, Price DL, Martin LJ. Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. *J Comp Neurol*. 1997; 378:70–87. [PubMed: 9120055]
- Sabir H, Scull-Brown E, Liu X, Thoresen M. Immediate hypothermia is not neuroprotective after severe hypoxia-ischemia and is deleterious when delayed by 12 hours in neonatal rats. *Stroke*. 2012; 43:3364–3370. [PubMed: 22996953]
- Salinas-Zeballos, ME.; Zeballos, GA.; Gootman, PM. A stereotaxic atlas of the developing swine (Suscrofa) forebrain. In: Tumbleson, ME., editor. *Swine in Biomedical Research*. Plenum Press; New York and London: 1986. p. 887-906.
- Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, Cotten CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, Jobe AH, National Institute of Child Health and Human Development Neonatal Research Network. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N Engl J Med*. 2005; 353:1574–1584. [PubMed: 16221780]
- Shankaran S, Pappas A, McDonald SA, Vohr BR, Hintz SR, Yoltan K, Gustafson KE, Leach TM, Green C, Bara R, Petrie Huitema CM, Ehrenkranz RA, Tyson JE, Das A, Hammond J, Peralta-Carcelen M, Evans PW, Heyne RJ, Wilson-Costello DE, Vaucher YE, Bauer CR, Dusick AM, Adams-Chapman I, Goldstein RF, Guillet R, Papile LA, Higgins RD, Eunice Kennedy Shriver NICHD Neonatal Research Network. Childhood outcomes after hypothermia for neonatal encephalopathy. *N Engl J Med*. 2012; 366:2085–2092. [PubMed: 22646631]
- Suh GJ, Kwon WY, Kim KS, Lee HJ, Jeong KY, Jung YS, Lee JH. Prolonged therapeutic hypothermia is more effective in attenuating brain apoptosis in a Swine cardiac arrest model*. *Crit Care Med*. 2014; 42:e132–42. [PubMed: 24145844]

- Tekes A, Poretti A, Scheurkogel MM, Huisman TA, Howlett JA, Alqahtani E, Lee JH, Parkinson C, Shapiro K, Chung SE, Jennings JM, Gilmore MM, Hogue CW, Martin LJ, Koehler RC, Northington FJ, Lee JK. Apparent diffusion coefficient scalars correlate with near-infrared spectroscopy markers of cerebrovascular autoregulation in neonates cooled for perinatal hypoxic-ischemic injury. *AJNR Am J Neuroradiol*. 2015; 36:188–193. [PubMed: 25169927]
- Wang B, Armstrong JS, Lee JH, Bhalala U, Kulikowicz E, Zhang H, Reyes M, Moy N, Spicer D, Zhu J, Yang ZJ, Koehler RC, Martin LJ, Lee JK. Rewarming from therapeutic hypothermia induces cortical neuron apoptosis in a swine model of neonatal hypoxic-ischemic encephalopathy. *J Cereb Blood Flow Metab*. 2015
- Wang L, Ma Q, Yang W, Mackensen GB, Paschen W. Moderate hypothermia induces marked increase in levels and nuclear accumulation of SUMO2/3-conjugated proteins in neurons. *J Neurochem*. 2012; 123:349–359. [PubMed: 22891650]
- Wegleiter K, Hermann M, Posod A, Wechselberger K, Stanika RI, Obermair GJ, Kiechl-Kohlendorfer U, Urbanek M, Griesmaier E. The sigma-1 receptor agonist 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) protects against newborn excitotoxic brain injury by stabilizing the mitochondrial membrane potential in vitro and inhibiting microglial activation in vivo. *Exp Neurol*. 2014; 261:501–509. [PubMed: 25111531]
- Xiong M, Cheng GQ, Ma SM, Yang Y, Shao XM, Zhou WH. Post-ischemic hypothermia promotes generation of neural cells and reduces apoptosis by Bcl-2 in the striatum of neonatal rat brain. *Neurochem Int*. 2011; 58:625–633. [PubMed: 21300124]

Highlights

1. Delayed hypothermia did not prevent white matter apoptosis in the developing brain after hypoxia.
2. Slow and rapid rewarming after delayed hypothermia promoted white matter apoptosis.
3. The apoptosis from hypothermia and rewarming can occur independent of hypoxic-ischemic injury.
4. The apoptosis after hypothermia and rewarming involves myelinating oligodendrocytes.
5. The rate of rewarming, 0.5°C/h or 4°C/h, did not affect the degree of apoptosis.

Fig. 1. Representative macrophotographs showing the anatomic regions where apoptotic profiles were counted in white matter at the striatal (A) and hippocampal (B) anatomic levels. Apoptotic profiles were counted in the subcortical white matter of the motor gyrus (i), corpus callosum (ii), first bundle of the internal capsule (iii), and lateral olfactory tract (iv). Photographs were taken at 10× magnification; grayscale images of hematoxylin & eosin stained sections are shown.

Fig. 2.

Normothermic sham and naïve unanesthetized piglets had similar apoptotic profile counts by hematoxylin and eosin (H&E) staining in the subcortical white matter of the motor gyrus at the striatal (A) and hippocampal (B) anatomic levels. TUNEL+ profile counts were also similar in the subcortical white matter of the motor gyrus at the striatal (C) and hippocampal (D) levels. The inset photos show a representative H&E apoptotic profile and a TUNEL+ profile. Photos were taken at 600× magnification with oil immersion.

Fig. 3. Representative images show histologic staining of brains from piglets that underwent sham surgery or hypoxia-ischemia injury (HI). The subcortical white matter of the motor gyrus is shown. (A) Hematoxylin and eosin (H&E) staining in a sham piglet without ischemic injury. The arrows identify examples of morphologically normal oligodendrocytes. Apoptotic cells were rarely observed in sham piglets. (B) H&E stain of an HI-injured piglet that received rapid rewarming. The arrows denote examples of apoptotic profiles. The inset shows apoptotic profiles at higher magnification. The arrowheads identify examples of normal

oligodendrocytes. (C) TUNEL assay in an HI-injured piglet that underwent rapid rewarming. The arrows identify TUNEL+ profiles, many of which had chromatin condensation characteristic of apoptosis. The inset shows TUNEL+ apoptotic profiles at higher magnification. (D) CNPase immunocytochemistry in an HI-injured piglet that was rapidly rewarmed. The arrow identifies a CNPase+ apoptotic oligodendrocyte. (E, F) H&E staining of brain tissue from an HI-injured, normothermic piglet shows normal glial morphology. Apoptotic cells were rare in piglets that received HI and normothermia. The arrows identify examples of morphologically normal oligodendrocytes. Photos A, B, C, and E were taken at 400× magnification, and the scale bar is 50 µm. The insets of panels B and C, and panels D and F were taken at 600× magnification with oil immersion; the scale bar is 10 µm. The TUNEL assay and immunocytochemistry in panels C and D were developed with 3,3'-diaminobenzidine substrate with cresyl violet counterstains.

Fig. 4.

Apoptosis in the subcortical white matter of the motor gyrus at the striatal (A, B) and hippocampal (D, E) anatomic levels. (A) At the striatal level, both hypoxia-ischemia (HI; $p=0.034$) and temperature ($p<0.001$) independently affected the H&E apoptotic profile counts. In post-hoc comparisons, HI and sham piglets that remained hypothermic (hypoT) or that received slow or rapid rewarming (RW) had more apoptosis than normothermic (normoT) piglets ($*p<0.05$ vs. HI normoT; $**p<0.05$ vs. sham normoT). Hypothermic shams had more apoptosis than rapidly rewarmed shams ($\dagger p=0.021$ vs. sham+rapid RW). Rapid rewarming after HI increased apoptosis above that observed in shams ($\dagger\dagger p=0.034$ vs. sham+rapid RW). During normothermia, HI increased apoptosis above that in sham piglets ($\#p=0.014$ vs. sham+normoT). (B) TUNEL stain demonstrated that temperature ($p<0.001$), but not HI ($p=0.539$), affected apoptosis, and there was a significant interaction between temperature and HI ($p=0.024$). In post-hoc comparisons, hypothermic, rapidly rewarmed, and slowly rewarmed shams had more TUNEL+ profiles than normothermic shams ($*p<0.001$ vs. sham+normoT). Hypothermia increased TUNEL+ counts more than rapid rewarming in shams ($**p=0.017$ vs. sham+rapid RW). Among HI piglets, hypothermia increased the number of TUNEL profiles above that in normothermia ($\dagger p=0.037$ vs. HI +normoT). Finally, normothermic HI piglets had more TUNEL+ profiles than did normothermic shams ($\dagger\dagger p=0.017$ vs. sham+normoT). (C) At the hippocampal level, H&E apoptotic profile counts were affected by temperature ($p=0.002$) but not by HI ($p=0.438$). Post-hoc comparisons showed that among sham piglets, both hypothermia and slow rewarming increased apoptosis above that in normothermic shams ($*p=0.040$ vs. sham

+normoT; ** $p=0.002$ vs. sham+normoT). (D) TUNEL profile counts did not show an effect of HI injury ($p=0.673$) or temperature ($p=0.060$) on apoptosis. Data are shown as means with SD. Analyses were conducted with 2-way ANOVA and post-hoc Holm Sidak pairwise comparisons.

Fig. 5.

(A) Representative images show histologic staining with H&E of white matter neurons and glia in the subcortical white matter of the motor gyrus. The arrows identify examples of white matter neurons, distinguished by the large cell body (characteristically 8-20 μm in diameter), multipolar or triangular neuronal morphology, open non-condensed nucleus with chromatin strands and often a nucleolus. The arrowheads show examples of the smaller glial cells, which include astrocytes and oligodendrocytes. The photo was taken at 400 $\times$ magnification, and the scale bar is 50 μm . The inset photo was taken at 600 $\times$ magnification with oil immersion. (B) Neuron counts in the subcortical white matter of the motor gyrus were affected by hypoxic-ischemic (HI) injury ($p=0.023$), but not temperature ($p=0.576$), by 2-way ANOVA. In post-hoc Holm-Sidak multiple comparisons, sham piglets had more white matter neurons than HI piglets after slow rewarming (RW; $*p=0.029$ vs. HI+slow RW). Neuron counts were similar between HI and sham piglets that received normothermia (normoT), hypothermia (hypoT), or rapid rewarming. When data were stratified by HI injury, neuron counts did not statistically differ by temperature. Data are shown as means with SD.

Fig. 6.

Apoptotic profile counts by H&E stain in the corpus callosum (A), lateral olfactory tract (B), and first bundle of the internal capsule (C). (A) Hypoxic-ischemic (HI) injury ($p<0.001$), but not temperature ($p=0.355$), affected apoptotic profile counts in the corpus callosum. In post-hoc comparisons, HI piglets that remained normothermic (normoT) or received slow or rapid rewarming (RW) had more apoptosis than sham piglets that received the same temperature treatment (* $p<0.001$ vs. sham+normoT; ** $p=0.002$ vs. sham+rapid RW; † $p=0.005$ vs. sham+slow RW). (B) In the lateral olfactory tract, HI increased apoptosis

independent of temperature management ($p=0.014$). Temperature also affected apoptosis independent of HI ($p=0.005$). After controlling for HI injury, hypothermic (hypoT) piglets had more apoptosis than did normothermic ($*p=0.018$), rapidly rewarmed ($**p=0.023$), and slowly rewarmed ($†p=0.012$) piglets. No significant post-hoc pairwise comparisons were identified when stratifying the data by injury or temperature. (C) Neither HI injury ($p=0.591$) nor temperature ($p=0.081$) affected apoptotic profile counts in the internal capsule. Data were analyzed using two-way ANOVA and post-hoc Holm-Sidak pairwise comparisons. Data are shown as means with SD.

Figure 7.

Levels of cleaved caspase-3 immunoreactivity in the subcortical white matter of the sensorimotor cortex. (A) Representative western blot with β-actin loading controls. (B) Western blots for cleaved caspase-3 in naïve, normothermic sham, hypoxia–ischemia +hypothermia (HI+HypoT), HI+rapid rewarming (HI+rapid RW), and HI+slow rewarming (HI+slow RW) groups were similar. Box plots with whiskers (5th to 95th percentiles) are shown.

Table 1

Physiologic variables of hypothermic and rewarmed sham piglets for histologic quantification of apoptotic profiles.

Parameter/Group	N	Baseline (mean±SD)	ROSC 1 h (mean±SD)	ROSC 3 h (mean±SD)	ROSC 20 h ^a (mean±SD)	ROSC 28 h (mean±SD)
Temperature (°C)						
Sham hypothermia	7	38.1 ± 1.3	38.9 ± 0.7	34.1 ± 0.4	33.9 ± 0.1	33.9 ± 0.2
Sham slow rewarm	7	37.8 ± 0.9	38.4 ± 0.8	34.5 ± 0.6	33.9 ± 0.1	38.1 ± 0.1
Sham rapid rewarm	8	38.5 ± 0.4	38.6 ± 0.3	34.5 ± 0.4	33.9 ± 0.2	38.7 ± 0.6
pH						
Sham hypothermia	7	7.45 ± 0.09	7.43 ± 0.09	7.45 ± 0.06	7.41 ± 0.05	7.40 ± 0.03
Sham slow rewarm	7	7.40 ± 0.08	7.46 ± 0.05	7.43 ± 0.06	7.43 ± 0.03	7.37 ± 0.04
Sham rapid rewarm	8	7.45 ± 0.07	7.48 ± 0.05	7.48 ± 0.08	7.43 ± 0.06	7.40 ± 0.07
PaCO ₂ (mmHg)						
Sham hypothermia	7	36 ± 5	41 ± 10	35 ± 4	39 ± 4	39 ± 6
Sham slow rewarm	7	37 ± 6	32 ± 4	32 ± 4	35 ± 3	38 ± 4
Sham rapid rewarm	8	38 ± 11	34 ± 6	31 ± 5	35 ± 5	40 ± 6
MAP (mmHg)						
Sham hypothermia	7	85 ± 14	75 ± 8	80 ± 13	76 ± 9	70 ± 7
Sham slow rewarm	7	73 ± 14	74 ± 12	84 ± 12	77 ± 13	75 ± 4
Sham rapid rewarm	8	77 ± 17	71 ± 13	83 ± 10	74 ± 13	63 ± 18
Hemoglobin (g/dL)						
Sham hypothermia	7	6.4 ± 1.6	6.9 ± 1.3	8.0 ± 1.6	8.3 ± 1.9	8.4 ± 2.0
Sham slow rewarm	7	7.6 ± 1.2	8.4 ± 1.0	9.9 ± 1.6	10.3 ± 2.4	9.4 ± 1.3
Sham rapid rewarm	8	7.7 ± 0.9	7.9 ± 0.9	8.9 ± 1.3	10.4 ± 1.9	8.8 ± 1.2
Sodium (mEq/dL)						
Sham hypothermia	7	143 ± 3	145 ± 3	144 ± 2	143 ± 4	146 ± 1
Sham slow rewarm	7	146 ± 6	144 ± 4	144 ± 3	141 ± 3	140 ± 3
Sham rapid rewarm	8	147 ± 7	149 ± 7	147 ± 7	142 ± 5	147 ± 5

ROSC, return of spontaneous circulation (ROSC 1 h refers to 1 h after ROSC); MAP, mean arterial blood pressure; PaCO₂, partial pressure of carbon dioxide.

^aPrior to initiating rewarming in rewarmed groups.

Table 2

Cell profile counts in the subcortical white matter.

Parameter/Group	N	H&E apoptotic profiles ^a (median; IQR)	N	TUNEL profiles ^a (median; IQR)	N	White matter neurons ^a (median; IQR)	N	H&E apoptotic profiles ^b (median; IQR)	N	TUNEL profiles ^b (median; IQR)
Sham normothermia	8	3 (2, 4)	8	4 (3, 7)	8	77 (74, 91)	8	5 (4, 14)	5	10 (9, 23)
Sham hypothermia	7	26 (16, 39)	7	30 (23, 37)	7	93 (76, 123)	7	11 (8, 37)	7	25 (16, 30)
Sham rapid rewarm	8	9 (8, 13)	8	17 (8, 24)	8	107 (87, 111)	NA	NA	NA	NA
Sham slow rewarm	7	20 (7, 30)	7	26 (13, 35)	7	101 (88, 111)	7	25 (18, 32)	6	32 (29, 36)
HI normothermia	6	6 (4, 9)	6	9 (7, 9)	6	84 (76, 89)	6	11 (4, 22)	3	17 (12, 26)
HI hypothermia	8	15 (13, 24)	7	21 (18, 27)	8	83 (78, 90)	8	18 (15, 20)	6	17 (14, 19)
HI rapid rewarm	8	20 (11, 29)	6	18 (13, 25)	8	74 (73, 96)	NA	NA	NA	NA
HI slow rewarm	8	19 (14, 24)	6	15 (13, 20)	8	85 (71, 90)	8	24 (14, 31)	6	22 (17, 27)

^a At the striatal anatomic level.

^b At the hippocampal anatomic level. NA, not applicable.

Apoptotic profiles counts by hematoxylin & eosin stain in the corpus callosum, lateral olfactory tract, and first bundle of the internal capsule.

Table 3

Parameter/Group	N	Corpus callosum (median; IQR)	Lateral olfactory tract (median; IQR)	Internal capsule (median; IQR)
Sham normothermia	8	5 (3, 7)	3 (1, 3)	1 (0, 2)
Sham hypothermia	7	8 (7, 22)	6 (4, 13)	4 (2, 4)
Sham rapid rewarm	8	5 (4, 7)	4 (1, 6)	10 (5, 11)
Sham slow rewarm	7	5 (4, 14)	3 (0, 5)	3 (1, 12)
HI normothermia	6	18 (17, 34)	5 (2, 9)	2 (0, 16)
HI hypothermia	8	13 (11, 23)	16 (8, 25)	12 (3, 19)
HI rapid rewarm	8	16 (11, 22)	6 (4, 9)	1 (0, 5)
HI slow rewarm	8	23 (13, 32)	5 (4, 12)	4 (2, 4)