Immediate Hypothermia Is Not Neuroprotective After Severe Hypoxia-Ischemia and Is Deleterious When Delayed by 12 Hours in Neonatal Rats

Hemmen Sabir, MD; Emma Scull-Brown; Xun Liu, PhD; Marianne Thoresen, MD, PhD

Background and Purpose—Hypothermia (HT) for neonatal hypoxic-ischemic encephalopathy is advised to start within the first 6 hours after birth. There is some clinical evidence that HT is more effective against moderate than against severe hypoxic-ischemic encephalopathy, but it is unknown whether delayed HT beyond 6 hours is effective or even injurious.

Methods—One-hundred seven 7-day-old rat pups underwent unilateral hypoxia-ischemia of moderate severity. Pups were randomized to receive 5 hours of normothermia (NT) or HT starting immediately, 3 hours, 6 hours, or 12 hours after the 90-minute hypoxic period. One-hundred five 7-day-old rat pups underwent severe hypoxia-ischemia lasting 150 minutes, followed by the same group design as mentioned. Relative area loss of the left/right hemisphere was measured after 1 week of survival.

Results—In the moderate NT group, the mean area loss of the left hemisphere was 40.5%. The area loss was significantly decreased to 24.8% with immediate HT (P<0.05) and increased linearly with the delay of HT by 1.788% per hour until at least 6 hours of delay (linear regression, P=0.026). After 12-hour delayed HT, the area loss was similar to the moderate NT group (41.1%). After severe NT, the mean area loss of the left hemisphere was 59.3%. Immediate HT, 3-hour delayed HT, and 6-hour delayed HT all resulted in similar area loss, whereas the 12-hour delayed-HT resulted in significantly increased area loss (69.5%; P=0.032).

Conclusions—Immediate and delayed (≤6 hours) HT provides neuroprotection after moderate hypoxia-ischemia in neonatal rats. This neuroprotection decreases linearly with increasing delay. After severe insults, however, immediate or delayed HT ≤6 hours provides no neuroprotection. Twelve-hour delayed hypothermia increased brain injury after severe hypoxia-ischemia, which is of clinical concern. (Stroke. 2012;43:00-00.)

Key Words: animal models ■ hypothermia ■ neuroprotection ■ pediatric neurology

Therapeutic hypothermia (HT) has become standard treatment for term newborns experiencing perinatal asphyxia. Randomized controlled trials have shown that HT significantly improves outcome after hypoxia-ischemia (HI). Meta-analysis of these suggests that HT improves outcome more in the group with moderate rather than severe HI encephalopathy. HI encephalopathy develops during different phases. Initial reperfusion restores cellular energy metabolism in ~30 to 60 minutes. A latent phase of 6 to 12 hours follows, in which cellular energy metabolism is fully or partially restored before a secondary decline in energy failure may occur. This secondary phase is characterized by excessive entry of Ca^{2+} into cells, free radical production, excitotoxic amino acid release, inflammation, and activation of apoptotic pathways, commencing within 24 hours and lasting days or weeks. These phases of injury and recovery are not strictly sequential, might overlap, and are likely to be changed by severity of injury or other conditions. As previously shown in different animal models, HT during reperfusion and the latent phase is neuroprotective. Clinical guidelines recommend that HT should be started within the first 6 hours after birth based on findings in a term-equivalent fetal sheep HI model showing significant neuroprotection when HT was initiated before 5.5 hours after cerebral injury. There are few studies published about the effect of HT initiated late after HI of different severity, and no study asks whether HT might increase injury if started very late. There might be species, age, and injury differences. In newborn pigs, delaying HT by 3 hours was ineffective. In adult rat stroke models, delayed treatment up to 3 hours is neuroprotective. Our aim was to investigate in moderate and severe unilateral brain injury in neonatal rats the effect of HT, the optimal duration of HT, and the effective time window of HT.

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One-hundred forty-two P7 rat pups of both sexes from 15 litters all underwent a left common carotid ligation under general anesthesia as previously described.15,25,26 After a maximum delay of 180 minutes, while pups were with their dam, pups were exposed to 8% oxygen for 150 minutes at Trectal of 37.0°C in a temperature-controlled chamber. This is an additional area reduction when HT was prolonged from 5 hours to 10 hours.

Materials and Methods

Procedures
All procedures were performed under Home Office license in accordance with United Kingdom regulations and approved by the University of Bristol Animal Ethical Review Panel.

Optimal Duration of Hypothermia Treatment
Previously, we found 5 hours of HT to be more effective than 3 hours in newborn rats,24 so it became our standard duration.25 In this current study, we compared the effect of 5 hours vs 10 hours of HT. Twenty-seven Wistar-rat pups (Charles River, Margate, UK; 7 days old [P7]) from 3 litters underwent left carotid ligation followed by moderate HI and were randomized for 5 hours with immediate HT, and 5 hours with immediate normothermia (NT) at Trectal 37.0°C. There was no significant increase in neuroprotection when HT was prolonged from 5 hours to 10 hours (Figure 1). All further experiments used 5 hours of HT. In addition, blood gases, blood glucose, and lactate levels did not significantly differ (Online Data Supplement).

Moderate Injury
One-hundred forty-two P7 rat pups of both sexes from 15 litters all underwent a left common carotid ligation under general anesthesia as previously described.15,25,26 After a maximum delay of 180 minutes, while pups were with their dam, pups were exposed to 8% oxygen for 90 minutes at Trectal of 36.0°C in a temperature-controlled chamber, which was described to result in moderate HI insult.25,26 Twenty-three pups died during this moderate insult. Twelve animals carrying a temperature probe were excluded from further analysis.25 One-hundred seven pups were randomized between different litters, matched for sex and weight (Table) to the following treatments for 5 hours in 21% oxygen: (1) immediate HT vs moderate NT (NT-Mod); (2) 3 hours with dam, followed by HT delayed 3 hours vs NT-Mod; (3) 6 hours with dam, followed by HT delayed 6 hours vs NT-Mod; and (4) 12 hours with dam, followed by HT delayed 12 hours vs NT-Mod. The temperature was continuously measured in additional “sentinel” pups in each chamber with a rectal temperature probe (IT-21; Physitemp Instruments) or a skin probe (CritiCool; MTRE) on the abdomen. Both probes were calibrated to ±0.1°C over a range of 20.0°C to 40.0°C against a certified mercury-in-glass thermometer (BS593; Zeal). For the NT group, Trectal of 37.0°C±0.2°C was maintained with a servo-controlled mat (CritiCool). Rectal temperature correlates within 0.1°C with brain temperature in P7 rats.18 For the HT group, Trectal of 32.0°C±0.2°C was achieved within 15 minutes. After the treatment period, pups were immediately removed from the chamber and returned to their dam. No clinical seizures were observed. While with their dam, the temperature of the probe animals was intermittently measured. All animals were kept in a 12:12 hours dark/light cycle at 22°C environmental temperature with adequate food and water and weights were checked daily.

Severe Injury
One-hundred sixty-eight P7 pups of both sexes from 14 litters all underwent a left common carotid ligation under general anesthesia as described. All pups were exposed to 8% oxygen for 150 minutes at Trectal of 37.0°C in a temperature-controlled chamber. This is an increase in time (150 vs 90 minutes) and temperature (37.0°C vs 36.0°C) compared with the moderate insult. A total of 121 pups survived, this insult, representing a severe HI insult with 28% mortality. Sixteen probe animals were excluded for further analysis and 105 pups were randomized between different litters, matched for sex and weight (Table) to the following treatments for 5 hours in 21% oxygen: (1) immediate HT vs severe NT (NT-Sev); (2) HT delayed 3 hours vs NT-Sev; (3) HT delayed 6 hours vs NT-Sev; and (4) HT delayed 12 hours vs NT-Sev. Temperature treatment was performed as described. After 5 hours of HT or NT, the pups followed the same protocol as the ones after the moderate injury.

Histopathology and Area Measurement
After 7 days of survival, transcardiac perfusion with 10% neutral-buffered formalin was performed under deep isoflurane/N2O anesthesia and brains were kept in 10% neutral-buffered formalin until

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<th>Table. Two Experimental Set-Ups</th>
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<td>Moderate Insult</td>
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<td>Weight at P7</td>
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<td>Severe Insult</td>
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<td>Weight at P7</td>
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<td>Weight gain at P14</td>
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Mean (±SD). There was no significant difference in weight at 7 days of age (P7) or in weight gain after 7 days of survival (P14). del indicates delay; HT, hypothermia; Imm, immediate; NT, normothermia; SD, standard deviation; Sev, severe.
further processing. Coronal 3-mm blocks were cut through the brain using a standard matrix for uniformity (ASI Instruments) and were embedded in paraffin. Blocks were cut into 5-μm sections and stained with hematoxylin and eosin. Two sections from each of 2 neighboring blocks representing cortex, hippocampus, basal ganglia, and thalamus were scanned with a scanner (Perfection V30; Epson) at 1200 dpi resolution. To measure the area of brain tissue loss, the image of each section was opened in ImageJ software (version 1.43, ImageJ; National Institutes of Health) by an individual blinded to the experimental treatment. A subset was examined by 2 blinded assessors. The midline of each brain section was identified on the image and the brain was divided by its hemispheres (left vs right). ImageJ was used to measure the area of viable tissue in the left and right hemisphere (Figure 2). The ratio of the measured brain area was calculated for the 2 sections per brain and the average percentage of area loss was calculated, respectively (1–[area ratio (left vs right)]×100).27

We validated this computerized method against our neuropathology score18,25–26 with P14 rat brains, previously scored by a neuropathologist using our validated scoring system with a 9-step scale from 0.0 (no injury) to 4.0 (90% injury).26 A pathology score of ~50% correlated with a computer-calculated area loss of ~40%. A 60% area loss corresponded to ~3.5 to 4.0 pathology score (>80% injury). There was a significant correlation of percentage of area loss and percentage of injury using our ImageJ area analysis (r2=0.9; Figure 2). This simple method of area measurement was feasible and reproducible between blinded individuals.

Data Analysis

Statistical analyses were performed with SPSS version 18 (SPSS). For 2-group comparisons, the Wilcoxon test was used. One-way analysis of variance was used to compare the different treatment groups of our moderate and severe insult experiments followed by a nonparametric post hoc test (Tamhane) if the analysis of variance showed no difference in the 1-way analysis of variance between the NT groups (P=0.644) was found, so the 4 NT groups were pooled and used as 1 NT-Mod group for further analysis. The NT groups showed no difference in injury despite the different regimes regarding when pups were separated from their dam.

The different results between the groups regarding percentage of area loss are shown in Figure 4A. The mean area loss in the pooled NT-Mod group was 40.5% (~12.3%; not significant). This significant reduction in area loss in the HT-treated animals decreased linearly with the delay by 1.785% per hour until at least 6 hours of delay (linear regression, P=0.026). HT with 12-hour delay gave no reduction in area loss compared with the NT-Mod group (Figure 4A).

Severe Injury

Figure 3B shows scatter plots of percentage area loss of the 4 NT-Sev groups (immediate NT, 57%; NT with 3-hour delay, 59%; NT with 6-hour delay, 61%; NT with 12-hour delay, 63%). No significant difference in 1-way analysis of variance analysis (P=0.96) was found and the 4 NT groups were pooled and used as 1 NT-Sev group in further analysis.
The different results between the groups regarding percentage of area loss are shown in Figure 4B. The mean area loss in the pooled NT-Sev group was 59.3% (±15.99). The mean area losses in the different treatment groups were: immediate HT, 56.8% (±15.60; not significant); HT with 3-hour delay, 59.4% (±20.13; not significant); HT with 6-hour delay, 61.6% (±7.93; not significant); and HT with 12-hour delay, 69.5% (±7.00; P=0.026). There was no significant 2-group difference between the NT-Sev group and immediate HT, HT with 3-hour delay, or HT with 6-hour delay groups, meaning there was no protection by HT with or without a delay. There was, however, a significant 10.2% increase in mean area loss in the HT with 12-hour delay group compared with the NT-Sev group (P=0.026). One-way analysis of variance showed a significant difference between the 5 treatment groups, and post hoc analysis (Tamhane) showed that the HT with 12-hour delay group was significantly different from the NT-Sev group (P=0.032). Figure 5 shows representative hematoxylin and eosin–stained sections for the severe injury groups.

**Discussion**

The main findings of this study are that immediate HT provides neuroprotection only after moderate HI in neonatal rats and that delayed HT by 12 hours increased brain injury after severe HI. After a moderate insult, protection decreases linearly with the delay of the start of cooling.

The neuroprotective time window is suggested to be within the first 6 hours after HI. Gunn et al. showed significant neuroprotection in their term-equivalent fetal sheep model of cerebral ischemia when HT was delayed for 5.5 hours compared with 8.5 hours. Similar to the findings by Gunn et
al, our linear regression analysis predicts no neuroprotection after 9 hours of delayed HT.

As experimentally shown, it is important to start neuroprotective treatments within the latent phase after HI encephalopathy. In a clinical setting, it is very unlikely that one knows the exact timing of the HI insult. Furthermore, there is sometimes a delay before signs of encephalopathy are recognized, and some infants have long transport times before reaching a neonatal intensive care unit. Hence, interventions are often started after 6 hours.

The Vannucci rat model of HI encephalopathy is a standardized model to assess brain injury and to examine neuroprotective strategies. The injury variability within the model is wide; therefore, large treatment groups have to be used. However, this experimental weakness strengthens the translational value of the model, because it reproduces variability observed in human neonatal HI. Because the brains in different species are very different in neuronal maturation, neuronal growth spurt, and vulnerability to HI, it is important to compare findings between species. We aimed to define the window for HT neuroprotection in neonatal rats. Using a moderate insult with increasing delay of HT as in the study by Gunn in fetal sheep, we also found immediate HT to be most neuroprotective, with a subsequent reduction until 9 hours of delay. These similar results in the 2 studies indicate that timing of cellular mechanisms and damage after HI encephalopathy are comparable between these animal species. Although the effect of the duration of HT seems to be different, Gunn found that 24 hours of HT was less effective than 72 hours. We did not find additional neuroprotection when we prolonged HT from 5 hours to 10 hours.

Meta-analysis of the first 3 cooling randomized controlled trials did not find any significant interaction between severity of encephalopathy and effectiveness of HT. The CoolCap Trial found protection only in those two-thirds of cooled newborns with moderate injury. In the current study, we examined moderate and severe injuries separately. We found that immediate HT after severe HI is not neuroprotective in neonatal rats. Of concern, starting HT after a 12-hour delay significantly increases brain injury in this severely injured group. Currently, it may not be possible with certainty to identify which newborns will have development of significant encephalopathy until some hours after birth. If these experimental results

![Figure 4. A, Graphs showing outcome (% area loss of left hemisphere after 1-week survival) of 7-day-old rat pups (P7) subjected to a moderate insult and randomized to 5 hours of immediate hypothermia (HT), HT with 3-hour delay, HT with 6-hour delay, and HT with 12-hour delay against an individual moderate normothermia (NT-Mod) group. Significant reduction in area loss in the immediate HT and HT with 3-hour delay groups compared with the NT-Mod group (P<0.05) and borderline significant reduction in the HT with 6-hour delay group (P=0.055). No reduction in area loss in the HT with 12-hour delay group. Black dots represent results of our predicted area loss based on linear regression analysis. Black line shows linear increase of area loss with the delay by 1.785% per hour until at least 6 hours of delay (linear regression, P=0.026). Based on the regression, HT was predicted to be protective up to 9 hours of delay. HT with 12-hour delay gave no reduction in area loss. B, Graphs showing outcome of P7 rats subjected to a severe insult and randomized to 5 hours of immediate HT, HT with 3-hour delay, HT with 6-hour delay, and HT with 12-hour delay against an individual severe NT (NT-Sev) group. No significant difference between the NT-Sev group and immediate HT, HT with 3-hour delay, or HT with 6-hour delay, meaning there was no protection by HT (P>0.05). There was a significant increase in mean area loss in the HT 12-hour delay group compared with the NT-Sev group (P=0.028). Values are mean ±1 standard deviation; *significance level of P<0.05.](http://stroke.ahajournals.org/content/2017/1/596007/figure-04)
are transferable to humans, then the therapeutic time window for HT treatment should not be extended.

In our previous neuroprotection studies, brains were assessed and scored by 2 pediatric neuropathologists. In this study, we show that computerized area measurements correlate well with our global neuropathology score. The computerized area assessment is fast, easy, and reproducible, but it is not possible to detect minor brain injury that would not result in significant area loss. This is reflected in a higher score on neuropathological examination than in the automated area measurement.

Survivors of severe asphyxia have development of severe motor and cognitive disabilities. Because we are not always able to identify the severely asphyxiated newborns early and confidently, the finding of our study is important when considering late HT. Some authors are suggesting that there might be a benefit from late cooling and increased depth or duration of cooling, and there is currently a trial to investigate this question clinically (Late Hypothermia for Hypoxic-Ischemic Encephalopathy, Clinical-Trials.gov, identifier: NCT00614744).

Additional neuroprotective treatments are of current research interest. Promising drugs producing an additive neuroprotective effect with HT are erythropoietin and xenon. In the world’s first feasibility study of adding 50% xenon to HT, we showed that its delivery is safe and feasible for 18 hours to asphyxiated newborns (Thoresen M, Liu X, Tooley J, Chakkarapani E, Dingley J, unpublished data 2011). Two small, phase 2 trials are ongoing (Xenon and Cooling Therapy in Babies at High Risk of Brain Injury Following Poor Condition at Birth [CoolXenon2], Clinical-Trials.gov, identifier: NCT01545271; and Neuroprotective Effects of Hypothermia Combined With Inhaled Xenon Following Perinatal Asphyxia [TOBYXe], Clinical-Trials.gov, identifier: NCT00934700). In addition, 1 randomized controlled trial is currently ongoing to assess the efficiency of erythropoietin in asphyxiated term newborns (Neonatal Erythropoietin in Asphyxiated Term Newborns, Clinical-Trials.gov, identifier: NCT00719407).

Conclusions
In conclusion, we have shown that 5 hours of HT at 32.0°C is the optimal HT duration in neonatal rats, and that up to 6-hour delayed HT provides neuroprotection after moderate but not severe HI. Of concern, late HT after 12 hours of delay in severely injured rats increased brain injury compared with NT. These experimental findings are extremely important and merit additional preclinical work.

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Disclosures
None.

References
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Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2012/09/20/STROKEAHA.112.674481.DC1
SUPPLEMENTAL MATERIAL

Immediate Hypothermia is Not Neuroprotective After Severe Hypoxia-Ischemia and Deleterious When Delayed by Twelve Hours in Neonatal Rats

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<th>5h HT</th>
<th>10h HT</th>
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<td><strong>pH</strong></td>
<td>7.39 (±0.02)</td>
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<td><strong>Blood glucose</strong></td>
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<td>3.77 (±0.63)</td>
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<td>[mmol/l]</td>
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<tr>
<td><strong>Blood lactate</strong></td>
<td>0.98 (±0.13)</td>
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**S 1:**

Descriptive table of the pH, blood glucose and blood lactate results of P7 rat pups, treated with HT for 5h (n=8) or for 10h (n=9) after HI. There was no significant difference between the pH, blood glucose and blood lactate levels of rat pups cooled for 5h or 10h. Results are presented as mean (±SD).