Long-Term Neuroprotective Effect of Postischemic Hypothermia in a Neonatal Rat Model of Severe Hypoxic Ischemic Encephalopathy: A Comparative Study on the Duration and Depth of Hypothermia

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ABSTRACT: It is not known whether deeper or longer hypothermia (HT) can achieve better neuroprotection against hypoxic ischemic encephalopathy (HIE) in neonates. To compare the neuroprotective effects of different durations and temperatures of postischemic HT in neonatal rats with severe HIE, 7-d-old rats were subjected to the Rice-Vannucci model for 150 min hypoxia. Only the rats with identified brain lesions in diffusion-weighted MRI were assigned to normothermia (NT, 36°C/48 h) or four HT (HT-30°C/48 h; HT-30°C/24 h; HT-33°C/48 h; and HT-33°C/24 h) groups. ¹H-magnetic resonance spectroscopy (¹H-MRS) and T2-weighted MRI were obtained serially, and functional studies were performed. HT groups showed significantly greater residual hemispheric volume and better rotarod and cylinder tests than did the NT group at 5 wk postischemia. HT groups also showed lower lactate-plus-lipid level in ¹H-MRS than did the NT group at 7 d postischemia. All of these outcome variables, however, did not differ among the 4 HT subgroups, despite a trend toward greater residual brain volume in the 48-h HT *versus* 24-h HT subgroups. In conclusion, neither reducing the target temperature from 33 to 30°C nor prolonging the duration from 24 to 48 h produced further improvements in neurologic outcomes in neonatal rat with HIE. (*Pediatr Res* 68: 303–308, 2010)

Hypothermia (HT) is one of the most promising neuroprotective modalities against hypoxic ischemic encephalopathy (HIE) in neonatal brain (1). However, in neonates with severe HIE, little neuroprotection could be achieved using current HT protocols in which whole-body or head cooling is performed for 48 to 72 h by maintaining the core temperature within a range of 33.5 to 34.5°C (2–5). In animal studies, the efficacy of postischemic HT was also limited in the subgroup with severe ischemic injury (6,7). To treat severe HIE by HT, a recent review suggested a “deeper” and “more prolonged” cooling (8). However, no controlled clinical trials have tested this supposition and several experimental studies failed to clearly demonstrate an effect of postischemic HT protocols with different target temperatures or duration in a perinatal HIE model (9–11).

The 7-d-old rat with unilateral carotid artery ligation and subsequent exposure to 8% hypoxia is the most commonly used model to evaluate the efficacy of therapeutic interventions in the developing brain (12), and several studies have used this Rice-Vannucci model (RVM) to test the effects of HT (10,11,13–19). However, an experimental design composed of a shorter duration of hypoxia, representing “mild” ischemic lesions, is not appropriate for evaluating the neuroprotective effect of HT against severe HIE. To date, few experimental studies have used the RVM with hypoxia exposure longer than 120 min (10,13,19), but none of these have reported the long-term neuroprotective effect of post-ischemic HT.

The aim of this study was to investigate the long-term neuroprotective effect of various postischemic HT protocols with different target temperatures and duration in a neonatal rat model of severe HIE.

METHODS

Animal model of HIE. Animal experiments were performed in accordance with the National Institutes of Health guidelines for humane handling of animals and were approved by the Committee of Animal Research of Asan Medical Center. Surgical procedures were performed on postnatal day 7 male Sprague-Dawley rats (*n* = 130, 10 litters). Rats were subjected to a well-characterized RVM (12). Briefly, rat pups were anesthetized with isoflurane gas (induction, 4.0%; maintenance, 1.0%) in oxygen:nitrous oxide (1:1). The right common carotid artery was ligated using 5/0 silk following midline incision. After 1 h recovery period, the pups were placed in a chamber maintained at a temperature of 36°C and exposed to humidified 8% oxygen for 150 min. After 15-min recovery period, diffusion weighted images (DWIs) and ¹H-magnetic resonance spectroscopy (MRS) were obtained. The time for the initial imaging study was about 15 min for each pup (total time for one experimental set <3.5 h). Only those rats with identified unilateral brain lesions in DWIs were assigned to the study groups of temperature intervention and the final studies of outcome measurement. Rats without apparent brain lesions or with severe lesions extending to the contralateral hemisphere were not included in the study and returned to their mothers until euthanized. After anesthesia, sham animals underwent exposure of the common carotid artery without ligation. During the intervention period, these rats, like the animals in the NT groups, were separated from their mothers and intermittently gavage-fed.

Received March 8, 2010; accepted June 18, 2010.

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Supported by a Grant (2008-10465) from the Asan Institute for Life Sciences, Seoul, Korea.

Abbreviations: DWI, diffusion-weighted image; HIE, hypoxic ischemic encephalopathy; HT, hypothermia; MRS, magnetic resonance spectroscopy; NAA, *N*-acetyl aspartate; NT, normothermia; RVM, Rice-Vannucci model; T2WI, T2-weighted image

Postischemic temperature modulation. Rats were classified into a sham group, a normothermia (NT) group (36°C/48 h) and four HT groups: HT-33/24 h (33°C/24 h), HT-30/24 h (30°C/24 h), HT-33/48 h (33°C/48 h), and HT-30/48 h (30°C/48 h). For temperature intervention, pups were separated from their mothers and placed in a temperature-controlled chamber within which each pup was separated from the others by an acrylic lattice to prevent temperature elevation through gathering. For groups of HT-30 and HT-33, temperature inside the chamber was initially set at 27.0 and 31.0°C, respectively, with minimal adjustment throughout the intervention period. By using a flexible temperature probe, rectal temperature was measured at 1 and 4 h, and thereafter every 4 h until the end of each intervention period. Through the period of temperature intervention, pups were fed five times per day with 0.4 mL of commercially available ready-to-feed formula by using 22G-animal feeding needle with round (15). After temperature intervention, the pups were returned to their dams until the end of imaging studies and functional studies at 5 wk postinsult.

MRI and ¹H-MRS. Animals were maintained under anesthesia with 1% isoflurane in a 1:2 mixture of O₂:N₂O. Images and MRS data were obtained using a 4.7-T MRI/MRS system (Bruker BioSpin, Karlsruhe, Germany) with a custom-made 25-mm diameter animal microimaging coil. Axial slices corresponding to coronal images in the neuroanatomic axis were collected from the cervical spinal cord to the olfactory bulb. MRIs and ¹H-MRS images were obtained at assigned times as follows: 1) immediately after injury (d 0), DWI/¹H-MRS; 2) 2 d after injury (d 2), T2-weighted images (T2WI)/¹H-MRS; 3) 7 d after injury (d 7), T2WI/¹H-MRS; and 4) 35 d after injury (d 35), T2WI. Images at d 2 were obtained after completion of temperature intervention. The imaging protocol included a DWI [TR = 2000 ms; TE = 76 ms; slice thickness = 1.5 mm; matrix = 128 × 64; field of view = 20 × 20 mm; and diffusion *B* value = 2217.7 s/mm² (no gap)], and a T2WI [TR = 4500 ms; TE = 80 ms; slice thickness = 1.5 mm; and matrix = 256 × 256 (no gap)]. The spectra from ¹H-MRS were acquired through a signal voxel (3 × 3 × 3 mm³) in the right parietotemporal region using a spin-acquisition mode sequence for 128 acquisitions with a TR of 3000 ms and a TE of 30 ms. All raw spectroscopic data were processed using the XWIN-NMR software provided with the scanner. The integrated area of the following metabolite peaks was recorded: *N*-acetyl aspartate (NAA) at 2.0 ppm; lactate-plus-lipid at 1.3 ppm; and lipid at 0.9 ppm. The relative sizes of the peak areas for each metabolite were quantified based on creatine levels at 3.0 ppm by an examiner blind to the study group.

Measurement of extent of brain injury. Volumetric analyses of MR images were performed using ParaVision 2.0.2 (Bruker, BioSpin, Karlsruhe, Germany). The boundary of each lesion was identified by a well-demarcated hyperintense area in T2WI at d 7 and d 35. To compensate for the effect of brain edema or shrinkage, the percent of the "intact" volume of the affected ipsilateral relative to the unaffected contralateral hemisphere was used as the outcome parameter; in each coronal image, the intact area of the ipsilateral hemisphere (*i.e.* the hemispheric area excluding the regions of hyper-intensity representing infarct or liquefaction) was outlined manually by two examiners blinded to group assignment. The size of the selected area was calculated automatically using the same software program. The residual brain volume of the ipsilateral hemisphere was calculated by summing the values of the intact area from six consecutive 1.5-mm-thick axial slices obtained caudally from the bregma level. Each of the six slices corresponded to coronal plate numbers 17, 24, 31, 38, 44, and 49 in the adult rat brain atlas of Paxinos (20). The volume of the contralateral hemisphere was calculated in the same manner. The percent of residual brain volume was calculated as (ipsilateral residual hemispheric volume/intact contralateral hemisphere volume) × 100. The values calculated by the two examiners was averaged and used in the final data analysis. Because the margin of the brain edema in MRIs obtained at d 0 and d 2 was blurred, we could not calculate the volume of brain edema. Instead, the extent of brain edema in DWIs at d 0 was categorized roughly into moderate (confined to the cortex, but without apparent lesions in the striatum or hippocampus) or severe (extension beyond the cortex into the striatum, thalamus, or hippocampus) injury by an examiner blinded to the study outcome (21).

Functional studies. Two functional tests were performed to evaluate sensorimotor function at d 37 by an examiner blind to the study group.

The length of time that a rat remained on the rotarod treadmill was measured (latency to fall). The rotation speed was accelerated from 4 to 40 rpm over 100 s for a maximum of 5 min. The average latency to fall from three trials, with a 15-min intertrial interval, was used as the measured parameter.

Asymmetry of forelimb use was determined by analyzing the movements of each rat during exploratory activity within a transparent polyacrylate cylinder (20 cm in diameter and 34 cm in height) with two mirrors positioned behind the cylinder. The forepaw placement of each weight-bearing contact with the wall during a full rear was recorded; "bilateral" was recorded in cases

of simultaneous contact with both forepaws. Each animal was video-recorded for 4 min in each session. Data are presented as the scores of limb-use asymmetry according to the following formula: [number of (left contacts + 1/2 of bilateral contacts)/number of (left + right + bilateral contacts)] × 100. This resulted in an asymmetry score that was lower for animals with poorer performance, *i.e.* decreased use of the affected (left) limb. All animals were recorded three times, and the average score of the trial(s) was recorded. For each animal, "inactive" trials (*i.e.* <12 wall movements per session) were excluded from the calculation of average scores.

Statistical analysis. χ^2 tests or Kruskal-Wallis tests were used to compare the mortality between the groups. *t* test was used in the quantitative analysis of the percent of residual volume and functional tests between two groups. The interobserver variability for percent of residual volume was assessed using Pearson's correlation coefficient. An ANOVA with Tukey's HSD test was used to compare the parameters among the four subgroups of HT. A repeated-measures ANOVA was used to compare ¹H-MRS values at d 0, d 2, and d 7, with each parameter (*e.g.* NAA) as a within-subject factor and study groups as the between-subject factor. The effects of the severity of initial brain injury and HT on final residual brain volume were assessed by two-way ANOVA. All statistical analyses were performed with SPSS 13.0 for Windows.

RESULTS

Survival rates and final weights. A total of 102 pups with identified brain lesions in d 0 DWIs were included in the study. The mortality rate did not differ between the HT (7/68) and the NT (5/34) groups during the course of the study period. Mortality among HT groups also did not differ: HT-33/24 h (1/17), HT-30/24 h (2/17), HT-33/48 h (0/16), and HT-30/48 h (4/18) ($p = 0.171$). Mean body weight at d 35 did not differ between the NT (182.5 ± 14.5 g) and the HT (179.3 ± 18.3 g) groups.

Maintenance of rectal temperature. Before temperature intervention, rectal temperature did not differ between the NT (35.8 ± 1.2°C) and the HT (35.6 ± 1.0°C) groups. From 1 h after initiating temperature control, rectal temperature within each group remained stable during the intervention period (24 or 48 h; Fig. 1).

Long-term neurologic outcomes. HT groups showed better outcomes than the NT group in the percent of residual volume at d 35 (sham, 98.8 ± 5.4%; NT, 42.2 ± 20.5%; and HT, 57.1 ± 21.4%), the rotarod test (sham, 176.7 ± 53.9 s; NT, 64.7 ± 37.0 s; and HT, 96.5 ± 50.4 s), and the cylinder test (sham, 48.3 ± 3.5%; NT, 31.4 ± 11.1%; and HT, 36.9 ± 10.1%) (Fig. 2). The concordance between both readers in determining the percent of residual volume at d 35 showed an excellent correlation coefficient ($R_p = 0.943$, $p < 0.001$). A

Figure 1. Measurement of rectal temperature in the NT (■) group and two representative HT subgroups (HT-30/48 h (●) and HT-33/48 h (▲)). Temperatures within each group remained stable during the intervention period and were significantly different among groups at each time point of measurement (* $p < 0.001$ by ANOVA).

Figure 2. Long-term neurologic outcomes in each study group. A, Percent of residual ipsilateral to contralateral hemispheric volume at d 35; B, rotarod test; C, cylinder test (* $p < 0.001$, ** $p < 0.01$, § $p < 0.05$ by t test).

Figure 3. Serial changes in ^1H -MRS data in the study groups. A, N-acetyl aspartate; B, lactate-plus-lipid; C, lipid (* $p < 0.05$ for HT vs. NT group). Mean ($\pm$ SEM) values (with error bars) for each parameter in sham (▲), NT (■), and HT (●) (* $p < 0.05$ for HT vs. NT group).

comparative analysis showed an excellent correlation between residual hemispheric volume at d 7 and d 35 ($R_p = 0.922$, $p < 0.001$). The residual hemispheric volume ratio at d 35 was significantly correlated with the results of rotarod test ($R_p = 0.333$, $p < 0.001$) and cylinder test ($R_p = 0.386$, $p < 0.001$).

^1H -MRS study. At d 0, while the levels of NAA did not differ among the sham (0.84 ± 0.12), the NT (0.91 ± 0.15), and the HT (0.89 ± 0.17) groups, the level of lactate-plus-lipid was significantly higher in the NT (1.38 ± 0.38) and the HT (1.39 ± 0.40) groups compared with the sham (0.72 ± 0.24) group. After temperature intervention, both NT and HT groups showed lower levels of NAA and higher levels of lactate-plus-lipid and lipid than the sham group at each time point (Fig. 3). Repeated-measures ANOVAs between the NT and the HT groups revealed a significant change in lactate-plus-lipid ($p < 0.01$) and lipid ($p < 0.001$) levels at each time of measurement after hypoxic injury. NAA levels did not change significantly over the course of the experiment. HT was effective in reducing the increased level of lactate-plus-lipid after HIE ($p < 0.05$). At d 7, the level of lactate-plus-lipid in the HT group (1.50 ± 0.83) was significantly lower

than that in the NT group (2.02 ± 1.11); lipid levels were also lower in the HT group (1.09 ± 0.52) than in the NT group (1.43 ± 0.69). NAA levels did not differ between the NT (0.83 ± 0.19) and HT (0.87 ± 0.17) groups.

Effect of depth and duration of HT on neurologic outcome. Subgroup analyses between the two subgroups with different target temperature but the same HT duration or between the two subgroups with different duration but the same target temperature revealed no differences in the residual hemispheric volume or functional studies at 5 wk postinsult (Table 1). Although residual brain volume ($60.5 \pm 22.3\%$ versus $53.8 \pm 20.3\%$) and cylinder test ($38.9 \pm 11.1\%$ versus $34.9 \pm 8.7\%$) results trended higher in the two 48 h-HT subgroups compared with the two 24 h-HT subgroups, these differences did not reach statistical significance.

Subgroup analyses of ^1H -MRS results between groups with different duration but the same target temperatures revealed a greater level of NAA in the subgroups treated with 48 h of HT at both 33°C ($p < 0.001$) and 30°C ($p < 0.05$) at d 2. Accordingly, as expected, the level of NAA was greater in the 48-h HT subgroups (0.90 ± 0.18) than in the 24-h HT

Table 1. Comparison of neurologic outcomes in the four HT subgroups

Parameters	33/24 h (n = 16)	30/24 h (n = 15)	33/48 h (n = 16)	30/48 h (n = 14)	p
Residual brain volume (%)*	52.4 ± 21.7	55.4 ± 19.3	61.4 ± 20.1	59.4 ± 25.4	NS
Rotarod test (s)	87.1 ± 44.6	109.9 ± 68.2	106.7 ± 50.3	81.1 ± 28.4	NS
Cylinder test (%)	35.1 ± 10.4	34.6 ± 7.1	37.0 ± 10.6	40.9 ± 11.7	NS
N-acetyl aspartate					
d 0	0.84 ± 0.18	0.87 ± 0.19	0.94 ± 0.14	0.92 ± 0.14	NS
d 2	0.68 ± 0.18	0.73 ± 0.20	0.95 ± 0.21	0.86 ± 0.13	NS
d 7	0.85 ± 0.18	0.82 ± 0.14	0.92 ± 0.18	0.91 ± 0.15	NS
Lactate-plus-lipid					
d 0	1.20 ± 0.45	1.52 ± 0.38	1.36 ± 0.30	1.51 ± 0.38	NS
d 2	2.31 ± 1.14	1.82 ± 0.75	2.08 ± 1.12	1.96 ± 1.11	NS
d 7	1.50 ± 0.86	1.60 ± 0.85	1.33 ± 0.57	1.58 ± 1.02	NS
Lipid					
d 0	0.69 ± 0.20	0.97 ± 0.32	0.86 ± 0.26	0.90 ± 0.32	NS
d 2	1.21 ± 0.41	1.12 ± 0.47	1.27 ± 0.67	1.23 ± 0.73	NS
d 7	1.06 ± 0.44	1.26 ± 0.58	0.95 ± 0.39	1.12 ± 0.64	NS

Data are presented as means ± SDs among the four HT subgroups, by ANOVA.

* Percent of ipsilateral intact hemispheric volume to contralateral hemispheric volume.

NS, not significant.

subgroups (0.70 ± 0.19) at d 2 ($p < 0.001$). Subgroup analyses performed as described earlier showed that neither lactate-plus-lipid nor lipid levels differed among subgroups at any measurement time.

Effect of severity of ischemic injury on neurologic outcome. On the basis of the results of the extent of the brain edema in DWIs at d 0, we categorized rats into moderate ($n = 52$) or severe ($n = 50$) initial injury groups. The ratio of rats with initial severe ischemic injury did not differ among groups: NT (19/34); HT-33/24 h (8/17); HT-30/24 h (8/17); HT-33/24 h (7/16); and HT-33/48 h (8/18). Two-way ANOVAs demonstrated that both the severity of ischemic injury and HT influenced several markers for long-term neurologic outcomes in neonatal rats with HIE, although not all changes were statistically significant (Figs. 4 and 5).

DISCUSSION

Postischemic HT with a duration of 24 h or longer and/or a target temperature of 33°C or lower, started within 3.5 h after

recovery, decreased infarct volume and improved sensorimotor functions in a neonatal rat model of severe HIE. This long-term neuroprotective effect of postischemic HT was previously demonstrated in several studies using neonatal rats (6,11,14–17). However, ours is the first study to investigate the effect of both duration and depth of HT in a neonatal rat HIE model.

Lowering the target rectal temperature from 33 to 30°C did not provide additional neuroprotection against severe HIE in the neonatal rat. This is in accord with the results of a few animal studies that focused on the effect of different HT target temperatures, although the temperature range varied among the studies (10,18–19,22). Interestingly, another piglet study demonstrated a different HT temperature threshold between brain regions; whereas HT at 35°C and 33°C achieved better neuroprotection in the deep gray matter and in the cortical gray matter, respectively, (9). Whether this differential neuroprotection afforded by different HT target temperatures also applies to the smaller brain of the neonatal rat is uncertain.

Figure 4. Two-way ANOVA demonstrating the dual effects of severity of initial ischemic damage (SI) and HT therapy on long-term neurologic outcomes. Mean values for moderate (Δ) and severe (□) initial damage groups. The severity of initial damage was based on the DWI at d 0 (see text). A, Percent of residual brain volume ($p < 0.001$ for SI; $p = 0.001$ for HT); B, rotarod test ($p < 0.01$ for SI; $p < 0.05$ for HT); C, cylinder test (not significant for SI; $p < 0.05$ for HT); D, N-acetyl aspartate at d 7 (not significant for SI or HT); E, lactate-plus-lipid at d 7 ($p < 0.001$ for SI; $p < 0.05$ for HT); and F, lipid at d 7 ($p < 0.001$ for SI; $p < 0.05$ for HT).

Figure 5. Evolution of brain lesions, as assessed by diffusion weighted (d 0) or T2-weighted (d 2, d 7, and d 35) MRIs in representative 7-d-old rat pups after right common carotid artery ligation and exposure to 8% oxygen for 150 min. Rats were categorized as having initially moderate (A) or severe (B) injury based on DWI findings obtained immediately after ischemic-insult and were treated with NT (*first row*) or HT (*second row*) for 48 h.

The subgroups treated with 48 h-HT did not clearly demonstrate better long-term neurologic outcomes than the subgroups with 24 h-HT. Although studies in adult animals have shown a beneficial effect of prolonged HT, the duration was markedly shorter than in current practice (23,24). Strategies that extend the period of HT beyond the therapeutic window generally target cells undergoing delayed neuronal death. In animal models of HIE, the phenotype of cell death varies depending on brain maturity, time, and the severity of the insult (25,26). Although necrosis is a major form of early neuronal death after HIE, both in neonatal and adult models, apoptosis plays a significant role in the delayed neuronal death that occurs 24 h after ischemic insult, especially in the developing brain (26–28). However, in RVM exposed to 2 h of hypoxia, the major pattern of neuronal death is still necrosis in most regions, particularly for the first 48 h postischemia (26). Moreover, the long-term contribution of delayed neuronal death to the final infarct volume is less significant than in rats exposed to 75 min of hypoxia (29). Delayed death of neurons remote from the ischemic core or from the penumbra (*e.g.* thalamus) has been found to be secondary to the loss of trophic support from the area of primary insult (29). Thus, in the case of a severe ischemic infarct in RVM, postischemic HT starting 24 h after the insult may have a lower probability of rescuing the neurons, not only in the ischemic core and penumbra but also in the deep brain regions, where death is due to target deprivation-mediated injury.

We also observed a trend toward increased residual brain volume and cylinder test results, as well as significant NAA preservation, after 48 h of HT. To extrapolate our results to clinical trials, it is important to consider differences between

neonatal rats and newborn infants in their stage of brain maturation, brain size, mode of ischemic insult, and cell death phenotypes. Unfortunately, we did not investigate whether further extension of HT duration to 72 h, the period of HT in clinical settings, results in better neuroprotection compared with shorter HT duration. There have been few animal studies of 72 h-HT (30,31) and none of these has systematically addressed issues regarding the duration of HT.

Contrary to ^1H -MRS profiles after HT in a piglet model of HIE (32), we found that signal levels at 1.3 ppm, representing lactate-plus-lipid, did not differ between the NT and HT groups at 2 d postinsult. Only at d 7, during the chronic phase of ischemic injury, the levels were lower in the HT than the NT groups. The persistently elevated signal at 1.3 ppm beyond 24 to 48 h postischemia was probably not due to the anaerobic production of lactate, but rather to lactate production by infiltrating phagocytic cells (33) or a signal overlap between lactate and lipids (34,35). These signals arise from neutral lipid droplets localized to the microglia/macrophages in the infarct area (36). The difference in the d 7 signal peak between the NT and HT groups at both 1.3 and 0.9 ppm likely represents the difference in the extent of brain infarct.

By roughly dichotomizing the severity of brain injury with DWIs, we demonstrated a neuroprotective effect of HT in neonatal rats with different degrees of HIE. Even in the subgroups with marked ischemic injury, HT showed long-term neuroprotection. This tool to identify and measure the extent of brain injury without sacrificing the subjects is valuable in designing studies to compare the efficacy of a given therapeutic intervention because even after the same period of hypoxic-exposure in the RVM, wide individual variations can exist in the extent of brain damage (25). The extent of lesions appeared in MRIs immediately after the insult well reflect the pathologic stage of the acute ischemic injury and also correlates well with the long-term histologic outcome (6,37). Unfortunately, poor resolution of DWIs obtained with shorter acquisition times failed to provide a quantitative measurement of the extent of the initial ischemic injury.

In conclusion, although postischemic HT decreased the extent of brain infarct and improved functional outcomes in 7-d-old rats with severe HIE, the benefit of longer or deeper HT was still not conclusive. However, the trend toward better neuroprotection of 48 h-HT *versus* 24 h-HT warrants additional comparative studies, with further extension of HT, in various models of HIE in the developing brain.

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