Topiramate Extends the Therapeutic Window for Hypothermia-Mediated Neuroprotection After Stroke in Neonatal Rats

YiQing Liu, MD; John D. Barks, MD; G. Xu, MD, PhD; Faye S. Silverstein, MD

Background and Purpose—Critical factors influencing the neuroprotective efficacy of postischemic hypothermia include depth, duration, and time of onset of cooling. In clinical practice, there is an unavoidable lag between the hypoxic-ischemic (HI) insult and the opportunity to initiate cooling. We hypothesized that early administration of a neuroprotective agent in combination with later-onset cooling could represent an effective therapeutic intervention after neonatal HI. We evaluated whether treatment with topiramate, a clinically available anticonvulsant, increased the efficacy of delayed post-HI hypothermia in a neonatal rat stroke model.

Methods—Postnatal day 7 (P7) rats underwent right carotid artery ligation followed by 1.5 hours of exposure to 8% oxygen. Fifteen minutes post-HI, animals received injections of topiramate (30 mg/kg) or PBS. Cooling was initiated 3 hours later (“delayed hypothermia”) in all animals (3 hours, in 27°C incubator). Functional outcome (forepaw response to vibrissae stimulation) and pathology (morphometric lesion measurements) were evaluated at P15 and P35.

Results—Neither topiramate nor delayed hypothermia alone conferred protection in this protocol. Combined treatment with topiramate and delayed hypothermia improved both performance and pathological outcome in P15 and P35 rats compared with PBS-treated animals that underwent delayed hypothermia concurrently. At P15, functional measures were better in topiramate-treated animals (mean correct forepaw response 9.3/10 versus 4.8/10; \(P<0.001\)), and there was >50% reduction in tissue loss (\(P<0.001\)); trends were similar at P35.

Conclusions—Our data provide the impetus for further evaluation of therapeutic approaches that combine drug therapy with delayed-onset cooling after neonatal HI brain injury. (Stroke. 2004;35:1460-1465.)

Key Words: cerebral ischemia ▪ cooling ▪ perinatal

Results of experimental and clinical studies are generating considerable interest in evaluation of hypothermia as a neuroprotective therapy after cerebral ischemia in children and adults. Critical factors that influence the neuroprotective efficacy of postischemic hypothermia include depth, duration, and time of onset of cooling.1

In clinical practice, there is an unavoidable lag between recognition of an hypoxic-ischemic (HI) insult and the opportunity to initiate cooling; this time interval may reduce the efficacy of treatment. We hypothesized that early administration of a neuroprotective drug in combination with later-onset cooling could represent an effective therapeutic intervention after neonatal HI. In this study, we evaluated whether administration of topiramate (TOP) increased the neuroprotective efficacy of delayed post-HI hypothermia in a well-characterized neonatal rat stroke model in which focal ischemic brain injury is elicited by combining unilateral carotid artery ligation with subsequent timed exposure to moderate hypoxia in postnatal day 7 (P7) rats.2 Factors that prompted us to evaluate TOP as adjunctive therapy included its efficacy in suppression of hypoxic seizures in a neonatal rodent model,3 its neuroprotective properties in adult cerebral ischemia models,4,5 and its clinical availability.

Previous work6 identified an important potential pitfall in analysis of outcome after post-HI hypothermia in this model, the phenomenon of apparent neuroprotection after a brief (eg, 1 week) recovery period, followed by recognition of progression of injury after a more prolonged (eg, 1 month) recovery period. To address this issue, we evaluated tissue damage at 1 and 4 weeks after lesioning.

Materials and Methods

Materials

TOP was provided by Johnson and Johnson Research and Development as a powder; a solution was prepared in PBS according to the instructions of the manufacturer before each experiment.

Surgery

Figure 1 summarizes the procedural timelines. P7 Sprague-Dawley rats (12 to 15g; Charles River Breeding Laboratories, Wilmington, Mass) were anesthetized with isoflurane (3.5% induction, 1.5% maintenance), and the right carotid artery was transected.2 One hour
later, animals were placed in warmed chambers (36.5°C) and exposed to 8% O₂/92% N₂ for 1.5 hours. Within 15 minutes after the end of hypoxia, animals received intraperitoneal injections of TOP (30 mg/kg, 120 to 150 μL) or an equivalent volume of saline. In preliminary experiments to evaluate TOP alone versus saline (n = 12/group), animals were then returned to the dams.

In experiments to evaluate the combination of drug treatment plus delayed hypothermia, a 3-hour delay before cooling was selected to model a clinically relevant time interval for initiation of this type of intervention. We first replicated a cooling protocol that, when initiated immediately, post-HI had no adverse effects on survival and improved histopathologic outcome at P14–P15 (3 hours, 27°C incubator); this protocol was used for delayed hypothermia treatment. In preliminary experiments, we found that treatment with delayed hypothermia alone did not confer protection on P15 (on the basis of functional testing and histopathology; data not shown).

In P7 rats, rectal temperatures accurately reflect brain temperatures. Animal temperatures were recorded before surgery, within 15 minutes after hypoxia, 3 hours later (pre-cooling), and 6 hours later (postcooling), using a 0.6-mm diameter flexible temperature probe, inserted rectally (thermometer 43T with probe 554; Yellow Springs Instruments). A practical issue that emerged was how to maintain body temperatures during the 3-hour post-HI recovery period. In initial experiments, animals recovered in incubators (set at 37°C); however, we noted that “nesting” temperatures (ie, physiological temperatures measured when pups were removed from their dams) were lower than temperatures measured after recovery in incubators (see Table 1). Therefore, in subsequent experiments in which long-term outcome was evaluated, animals recovered with the dams for 2.75 hours before cooling.

In one experiment, serum glucose was estimated at the end of hypothermia using a blood drop obtained by ear punch assayed by Chemstrips-BG (Boehringer), and mean (±SD) values were the same in both groups [n = 6/group; 65 ± 5 mg %].

Table 1. Serial Temperature Measurements

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<thead>
<tr>
<th>Protocol 1</th>
<th>Protocol 2</th>
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<tr>
<td><strong>TOP</strong></td>
<td><strong>PBS</strong></td>
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<tr>
<td>Presurgery</td>
<td>32.5±1.5</td>
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Values are means ± SD.
Protocol 1. Animals (n = 18/group) recovered in incubators (37°C) prehypothermia.
Protocol 2. Animals (n = 12/group) recovered with the dams prehypothermia.

Outcomes were compared in concurrently lesioned littermates that received TOP or PBS injections alone on P15. Then, outcomes were compared in concurrently lesioned littermates that received TOP or PBS injections followed by delayed hypothermia, both at P15, and also after a 4-week recovery period, on P35.

**Vibrisse Stimulation Test**

Although lesioned animals have no obvious motor impairment, sensorimotor testing can reveal asymmetrical function. We selected the vibrisse stimulation test because of its simplicity, sensitivity to deficits on P15, and ease of scoring; this task is primarily a measure of cortical sensorimotor function. The animal is held in the examiner’s hand, vibrisse are stimulated unilaterally on the edge of a horizontal surface (10 trials/ side; a normal response is immediate extension of the adjacent forepaw to that surface), and then appropriate limb placement is counted. Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals in Research.

**Histopathology**

Animals were killed by decapitation; brains were rapidly frozen in powdered dry ice. Every fourth frozen section (20 μmol/L) from anterior to posterior genu of corpus callosum was collected and cresyl violet stained. In P15 brains, 5 standard sections that encompassed the typical area of maximum damage were scanned and imported into NIH Image 1.61 software and areas of intact staining in cortex, striatum, and dorsal hippocampus were outlined and measured bilaterally. Percent damage values were calculated as 100×[(left–right)/left] area. In P35 brains, data were collected from 11 to 12 sections (640 μm apart); areas with intact staining were measured, and derived lesion volumes (left–right) and percent damage values were calculated.

**Data Analysis**

Between-group differences at P15 were evaluated by nonparametric testing. Two-way ANOVA was used to determine the effect of treatment on lesion volumes and on percent damage values at P35; post hoc analyses were applied to evaluate differences between specific data points. Statistical significance was set at P < 0.05.

**Results**

All animals survived surgery, drug treatment, and hypothermia. Two of 30 TOP+ delayed hypothermia (TOP-dHT)-treated and 1 of 30 PBS+ delayed hypothermia (PBS-dHT)-treated animals died subsequently. At P15 and P35, body weights did not differ between groups.

Table 1 summarizes results of sequential temperature measurements in all TOP-dHT and PBS-dHT animals; TOP had no effect on temperatures. Because temperatures before cooling were higher in animals that remained in an incubator during the initial recovery period (protocol 1) than in animals that were returned to the dam during this period (protocol 2),

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Figure 2 summarizes results of vibrissae stimulation testing at P15 in all TOP-dHT and PBS-dHT animals. In all animals, right-sided vibrissae stimulation consistently elicited right paw placement. In both groups of lesioned PBS-dHT-treated animals, there was marked impairment of left paw placement (L-placement) in response to left vibrissae stimulation, and performance was substantially better in both TOP-dHT groups (mean correct: 3.5/10 versus 8.7/10; *P < 0.001; Mann–Whitney tests).

In the protocol 2 groups, vibrissae stimulation testing was repeated on P35; trends were similar. There was marked impairment of left paw placement in the PBS-dHT group and minimal deficit in the TOP-dHT group (mean correct: 5.8/10 versus 8.7/10; *P < 0.001; Mann–Whitney test).

Figure 3 incorporates results from 2 sets of experiments, those in which the effect of TOP alone versus PBS were compared, and those in which TOP-dHT and PBS-dHT were compared. The data demonstrate the distributions of percent damage values in the 4 groups of lesioned animals at P15. Cross-sectional areas of cortex, striatum, and anterior dorsal hippocampus were measured in 6 standard sections per brain. Percent damage was calculated as 100 × (left − right)/left. Treatment with TOP alone did not influence tissue damage compared with PBS-injected littermate lesioned controls. In contrast, in the TOP-dHT-treated animals, in each region, injury was substantially reduced compared with PBS-dHT controls (*P < 0.005; Mann–Whitney tests); in 10 of 18 controls, there was >50% damage in at least 2 regions, whereas injury of this severity evolved in only 2 of 16 TOP-dHT-treated animals.

To evaluate whether neuroprotection was sustained, the experiments were replicated (incorporating prehypothermia recovery with dams [protocol 2]), and pathology outcome was evaluated 4 weeks later. Table 2 summarizes regional morphometry, derived lesion volumes, and percent damage values. Lesion volumes and percent damage values were reduced in the TOP-dHT group compared with PBS-dHT controls (*P < 0.001; 2-way ANOVA); post hoc Tukey tests demonstrated significant intergroup differences (*P < 0.01) in lesion volumes and percent damage values in each region analyzed.

Figure 4 compares histopathology at 3 corresponding levels in a PBS-dHT–treated animal and a TOP-dHT–treated animal at 4 weeks after lesioning. In the PBS-dHT control (Figure 4A through 4C), there is ipsilateral cerebral hemisphere atrophy, with prominent striatal atrophy (Figure 4A), and there is widespread cortical thinning (black arrowheads). Tissue infarction is limited to a focal area of posterolateral cortex, which is vulnerable to injury in this model. In this TOP-dHT animal (Figure 4D through 4F), there is minimal striatal and hippocampal tissue loss (7% damage, on the basis of morphometry); there is subtle cortical atrophy, and damage is also most pronounced posterolaterally.
Discussion

Our results demonstrate that early post-HI treatment with TOP in combination with delayed-onset cooling resulted in improved functional performance 1 and 4 weeks later and a sustained reduction in tissue injury in this neonatal rodent stroke model. Although each intervention had potential intrinsic neuroprotective properties, in this lesioning protocol, neither treatment alone was effective. The mechanism(s) underlying the efficacy of combination therapy in this setting is uncertain.

TOP conferred neuroprotection in adult rat models of global and focal cerebral ischemia. TOP has several modes of action that could contribute to attenuation of ischemic brain injury, including modification of sodium- and/or calcium-dependent action potentials, enhancement of GABA-mediated chloride fluxes into neurons, and inhibition of the AMPA/kainate type glutamate receptors [3–5,10]. TOP treatment was ineffective in the protocol that we evaluated. Because our primary goal was to identify an intervention that would enhance the efficacy of delayed-onset cooling, we did not systematically evaluate multiple TOP dose regimens, and it is possible that higher or repeated doses, or earlier drug administration, or both, could have improved outcome. The TOP dose selected was intermediate between 2 doses that were found to be neuroprotective in an adult middle cerebral artery occlusion model. A recent study in which the anticonvulsant properties of TOP were examined in neonatal rodents incorporated a much higher TOP dose (80 mg/kg). No information is available about blood or brain levels attained with either regimen. We selected a post-HI administration schedule because our priority was to attempt to model a clinical setting in which prophylactic treatment would not be feasible.

Technical limitations have precluded systematic analysis of electroencephalograms in this neonatal brain injury model; whether or not seizures exacerbate HI injury, both in this model and in the clinical setting of neonatal encephalopathy, remains an unresolved controversy. TOP administration can prevent posthypoxic seizures in immature rodents; however, we could not directly evaluate whether TOP prevented post-HI seizures in these experiments. Although prophylactic treatment with phenytoin before HI is neuroprotective in this model, post-HI treatment with the same dose is ineffective; phenytoin also has multiple modes of action, and its protective efficacy is not necessarily attributable to anticonvulsant properties. Chemically induced post-HI seizures did not amplify tissue injury in this model; however, these experiments did not directly address whether spontaneous seizures elicited by HI could contribute to progression of neuronal damage. In a fetal sheep model of perinatal asphyxia in which ischemia elicits intense epileptiform electroencephalogram activity 6 to 8 hours after insult, postischemic hypothermia attenuates injury only if it is started before the onset of seizures.

In this neonatal stroke model, there is substantial evidence that post-HI environmental and body temperature strongly influence the evolution of brain injury. Postischemic hyperthermia substantially exacerbates injury, and postischemic cooling can result in sustained decreases in neuronal damage. “Mild” hypothermia can be effective; longer periods of cooling (≥6 hour) are more effective. In contrast with the transient protective effects reported after brief (3-hour) post-HI hypothermia, longer (6- to 24-hour) cooling periods resulted in significant brain protection 5 weeks later. TOP, itself, had no effect on animal temperature.

### Table 2. Tissue Damage at P35

<table>
<thead>
<tr>
<th>Lesion Volume (mm³)</th>
<th>Percent Damage</th>
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<tbody>
<tr>
<td>Cortex</td>
<td>L</td>
</tr>
<tr>
<td>93±7</td>
<td>60±21</td>
</tr>
<tr>
<td>Striatum</td>
<td>35±2</td>
</tr>
<tr>
<td>22±6</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>5±3</td>
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<tr>
<td>32±2</td>
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<tr>
<td>28±20</td>
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**Volume (mm³)** | **Lesion Volume (mm³)** | **Percent Damage** |
<table>
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<tbody>
<tr>
<td>Cortex</td>
<td>87±6</td>
</tr>
<tr>
<td>Striatum</td>
<td>73±22</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>14±21*</td>
</tr>
<tr>
<td>Cortex</td>
<td>15±2</td>
</tr>
<tr>
<td>Striatum</td>
<td>14±3*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1±2*</td>
</tr>
</tbody>
</table>

Fifteen minutes post-HI, P7 animals (n = 12/group) received PBS or TOP; they were returned to their dams (2.75 hours), and then cooled (see Methods). Outcome was evaluated 4 weeks later (1 PBS-treated animal died). Coronal brain sections were cresyl violet stained; areas of intact staining were measured; regional volumes, lesion volumes and percent damage values were calculated. Values are means±SD. Lesion volumes and percent damage differed between groups (2-way ANOVA; Tukey post hoc tests indicated that values differed in each region (**P < 0.001**).
In preliminary experiments, we identified a delay period (3 hours) that resulted in a loss of efficacy of an established post-HI cooling period; it is conceivable that if cooling had been continued for a longer period, after this delayed onset, treatment efficacy would have been discerned, but such experiments were beyond the scope of this study. The primary objective of this study was to determine whether early post-HI drug therapy could restore the neuroprotective efficacy of delayed brief post-HI cooling at 1 week after lesioning, and our initial behavioral and pathology evaluation documented the efficacy of combination therapy to achieve this goal.

A noteworthy and puzzling observation in both neonatal and adult rodent models is that relatively brief (3 to 4 hours) posts ischemic hypothermia may delay the expression of rather than prevent brain injury (ie, at 3 to 7 days after the insult), neuronal damage appears to be attenuated but after a longer (1 to 2 months) recovery period, tissue damage progresses, and the initial protective effects of hypothermia are lost.6,18 One of the most surprising findings that emerged in our study was that protection conferred by the combination of TOP and brief delayed cooling was sustained for 1 month.

The cellular and molecular mechanisms that underlie the neuroprotective effects of posts ischemic hypothermia remain uncertain. Although the beneficial effects of intrinsically ischemic hypothermia can be readily attributed to a reduction in metabolic demands, there is no convincing evidence that this mechanism contributes substantially to the protective effects of modest posts ischemic cooling. Hypotheses invoked include less impairment of reuptake of the excitatory neurotransmitter glutamate, attenuation of nitric oxide synthase activity (resulting in reduced oxidative stress), inhibition of apoptosis, and inhibition of inflammation.1,19–21 Pharmacological inhibition of one or more of these pathways could have additive or synergistic protective effects in combination with posts ischemic hypothermia. One clearly defined example of synergism was provided by results of a study in which treatment with the antiinflammatory cytokine interleukin-10 in combination with posts ischemic hypothermia resulted in sustained neuroprotection after global ischemia in adult rats.19 It remains uncertain how TOP could amplify one or more of these mechanisms. Yet, it is interesting to note that there is increasing recognition that many diverse antiepileptic drugs have neuroprotective properties in the setting of brain ischemia.22

There are several ongoing clinical trials to evaluate induced hypothermia in term- asphyxiated neonates, and preliminary anecdotal reports are encouraging.23–24 The relevance of our data to the clinical setting is currently uncertain. The depth of cooling selected for these experiments was more extreme than would be clinically feasible in sick infants, and available data indicate that longer durations of more modest cooling would be a preferable clinical intervention. Future studies in larger animal models will be essential to address important therapeutic issues such as the role of anesthesia during cooling.25

In practice, the duration of the therapeutic window for initiation of cooling represents a critically important limitation, and if early drug treatment could extend this time interval, the opportunities for intervention could grow substantially. Our findings provide strong support both for the general hypothesis that combining neuroprotective drug therapy with posts ischemic hypothermia represents a potentially powerful therapeutic strategy,26 and more specifically, for the hypothesis that the feasibility of delayed-onset cooling in neonates could be enhanced if safe and effective combination treatment protocols were identified.

Acknowledgments
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References
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