Citicoline Treatment for Experimental Intracerebral Hemorrhage in Mice

Wayne Clark, MD; Lisa Gunion-Rinker, BS; Nikola Lessov, PhD; Kristin Hazel, BA

Background and Purpose—Citicoline sodium (cytidine-5′-diphosphocholine) has been shown previously to reduce ischemic injury in focal central nervous system models. Intracerebral hemorrhage (ICH) appears to be associated with an area of edema and ischemic injury surrounding the hematoma that may be reduced by neuroprotective therapy. The present study was designed to test whether treatment with citicoline reduces ischemic injury and improves functional neurological outcome in an experimental model of ICH.

Methods—In 68 Swiss albino mice (26 to 36 g), ICH was induced by collagenase injection into the caudate nucleus. Animals were randomized to receive either: citicoline 500 mg/kg or saline IP prior to collagenase and at 24 and 48 hours. Animals were rated on a 28-point neurological scale and sacrificed at 54 hours. The brains were sectioned, and the volume of hematoma, total lesion, and surrounding ischemic injury was determined.

Results—In terms of functional outcome, animals treated with citicoline had improved neurological outcome scores compared with placebo-treated animals: 10.4 ± 2.0 versus 12.1 ± 2.4 (P < 0.01). Regarding ischemic injury, although there was no difference in the underlying hematoma volumes, animals treated with citicoline had a smaller surrounding volume of ischemic injury than placebo-treated animals: citicoline, 13.8 ± 5.8 mm³ (10.8 ± 4.3% of hemisphere); placebo, 17.0 ± 7.1 mm³ (13.3 ± 5.1%) (P < 0.05).

Conclusions—In this animal model of ICH, treatment with citicoline significantly improved functional outcome and reduced the volume of ischemic injury surrounding the hematoma. This study supports a potential role for citicoline in clinical ICH treatment. (Stroke. 1998;29:2136-2140.)

Key Words: citicoline ■ intracerebral hemorrhage ■ treatment outcome

Primary intracerebral hematoma (ICH) is a major clinical problem, accounting for 15% of all acute stroke hospitalizations. Currently, there is no medical therapy available for these patients, with options being limited to supportive care or invasive neurosurgical evacuation.¹ There is a 35% mortality rate in patients with moderately sized ICH, with additional significant disability in many of the survivors.² Because ICH is an exclusion in the majority of ongoing acute stroke trials, it is unlikely that any medical therapy will be available in the near future. The damage induced by an ICH appears to be related to a combination of factors. There is a component of direct mechanical disruption from the hematoma. However, some of the surrounding injury also occurs secondary to edema formation and ischemia.³,⁴ Experimental studies have demonstrated a significant area of ischemia that surrounds the hematoma (penumbra).³,⁶ There is also a large area of edema, presumably mediated by glutamate release and free radical generation.⁵ It is therefore possible that agents that reduce ischemic stroke injury may also be beneficial in ICH.

Citicoline (cytidine-5'-diphosphocholine or CDP-choline) is an essential precursor for the synthesis of phosphatidylcholine, a key component of cell membranes. The exogenous administration of citicoline has been shown in animal models to reduce this cell membrane breakdown, leading to increased synthesis of phosphatidylcholine and decreased levels of free fatty acids. The use of citicoline treatment has been shown to be beneficial in several animal models of ischemia or hypoxia, including recent studies with reversible focal occlusion.¹⁸,¹⁹ These studies have found that citicoline treatment decreases free fatty acid concentration, improves neurological signs, decreases neurological deficits, restores animal learning performance, reduces glutamate-mediated injury, preserves phosphatidylcholine levels, and improves neuronal survival. Because citicoline treatment appears to protect neuronal tissue in the penumbra, it is likely that this therapy would prove beneficial in reducing the ischemia-related injury component of ICH. In the current study, we used a mouse adaptation of the collagenase hemorrhage model to test whether treatment with citicoline reduces ischemic injury and improves functional neurological outcome in an ICH model that approximates clinical ICH.

Received December 2, 1997; final revision received April 3, 1998; accepted July 21, 1998.
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Materials and Methods

Experimental Design
All animal procedures were approved by the Oregon Health Sciences University Institutional Review Board and are in accordance with guidelines published by the National Institutes of Health for animal use. Sixty-eight male Swiss albino mice weighing 26 to 36 g were anesthetized with halothane/O2 administered by an inhalation mask. Citicoline sodium (lot # 3D0397) was supplied by Interneuron Pharmaceuticals, Inc. The placebo was sterile saline. Upon arrival, citicoline was stored at room temperature in a desiccator. Animals were anesthetized with halothane/O2 administered by an inhalation mask. A 0.15-mL IP injection of ketamine (28 mg/mL), xylazine (2.8 mg/mL), and acepromazine (0.06 mg/mL) was injected over 2 minutes, with the needle left in place for 60 minutes. At 24 and 48 hours after ischemia, each mouse was anesthetized with halothane/O2 administered by an inhalation mask. Whole brains were mounted on a freezing microtome, and tissue sectioning occurred.

ICH Model
A 0.5-μL glass syringe filled with 0.9% saline was connected to PE 10 tubing and used to draw up the collagenase. A 30-gauge, 4-mm needle attached to the tubing was implanted into the caudate nucleus/globus pallidus (Stotnick and Leonard Atlas) at the coordinates AP-1.0, L-3.0, S-4.0, relative to stereotaxic zero. Bacterial collagenase (type VII-S, Sigma Chemical Co; 0.075 U in 0.5 mL) was injected over 2 minutes, with the needle left in place for an additional 3 minutes after injection. The mice were allowed to recover from surgery in a warm environment over a 3-hour period. Core animal temperature was maintained at 37±0.5°C during this time. Animals began to exhibit neurological signs of an ICH within 10 to 14 sections per brain were mounted on chrome-albumin-jelly slides. Slides were dried at room temperature overnight and then baked in a 37°C oven for a minimum of 6 hours. Sections were stained with Luxol Fast Blue (ICN Biomedicals Inc) and counterstained with Cresyl Violet Acetate (Eastman Kodak Co) to differentiate between the hematoma and the area of ischemia/edema around the hematoma. An NIH image analysis system with 1200 dpi flatbed scanner was used to measure the lesion areas. The following areas were identified on each section: total ipsilateral and contralateral hemisphere area, area of hematoma, total affected area (total lesion), and surrounding area of ischemic injury (ischemia plus edema; total lesion area minus hematoma area) (see the Figure). Volumes in cubic millimeters were calculated by multiplying the 0.5-mm slice thickness by the measured areas. To partially correct for effects of edema, all lesion areas were also expressed as percent of ipsilateral hemisphere involved. Results were analyzed with followed by 10% phosphate buffered formalin for 5 minutes (100 mL). Brains were then removed and placed in 7-mL scintillation vials containing 10% phosphate buffered formalin for postfixation overnight at 4°C. The formalin in each vial was replaced by 15% sucrose containing 0.05% sodium azide and stored at 4°C until sectioning occurred.

TABLE 1. Focal Deficits (0–28)

<table>
<thead>
<tr>
<th>Deficit (open bench top)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Body symmetry</td>
<td>Normal</td>
<td>Slight asymmetry</td>
<td>Moderate asymmetry</td>
<td>Prominent asymmetry</td>
<td>Extreme asymmetry</td>
</tr>
<tr>
<td>(2) Gait (open bench top)</td>
<td>Normal</td>
<td>Stiff, inflexible</td>
<td>Limping</td>
<td>Trembling, drifting, falling</td>
<td>Does not walk</td>
</tr>
<tr>
<td>(3) Climbing (gripping surface, 45° angle)</td>
<td>Normal</td>
<td>Climbs with strain, limb weakness present</td>
<td>Holds onto slope, does not slip or climb</td>
<td>Slides down slope, unsuccessful effort to prevent fall</td>
<td>Slides immediately, no effort to prevent fall</td>
</tr>
<tr>
<td>(4) Circling behavior (open bench top)</td>
<td>Not present</td>
<td>Predominantly one-sided turns</td>
<td>Circles to one side (not constantly)</td>
<td>Circles constantly to one side</td>
<td>Pivoting, swaying, or no movement</td>
</tr>
<tr>
<td>(5) Front limb symmetry (mouse suspended by its tail)</td>
<td>Normal</td>
<td>Light asymmetry</td>
<td>Marked asymmetry</td>
<td>Prominent asymmetry</td>
<td>Slight asymmetry, no body/limb movement</td>
</tr>
<tr>
<td>(6) Compulsory circling (front limbs on bench, rear suspended by tail)</td>
<td>Not present</td>
<td>Tendency to turn to one side</td>
<td>Circles to one side</td>
<td>Pivots to one side sluggishly</td>
<td>Does not advance</td>
</tr>
<tr>
<td>(7) Whisker response (light touch from behind)</td>
<td>Symmetrical response</td>
<td>Light asymmetry</td>
<td>Prominent asymmetry</td>
<td>Absent response ipsilaterally, diminished contralaterally</td>
<td>Absent proprioceptive response bilaterally</td>
</tr>
</tbody>
</table>

Tissue Preparation
At 54 hours, 6 hours after the final neurological scoring, each animal was anesthetized with a 0.15-mL IP injection of ketamine (28 mg/mL), xylazine (2.8 mg/mL), and acepromazine (0.06 mg/mL) and perfused transcardially with saline for 20 seconds (10 mL), followed by 10% phosphate buffered formalin for 5 minutes (100 mL). Brains were then removed and placed in 7-mL scintillation vials containing 10% phosphate buffered formalin for postfixation overnight at 4°C. The formalin in each vial was replaced by 15% sucrose containing 0.05% sodium azide and stored at 4°C until sectioning occurred. Whole brains were mounted on a freezing microtome, and tissue sectioning occurred. The following areas were identified on each section: total ipsilateral and contralateral hemisphere area, area of hematoma, total affected area (total lesion), and surrounding area of ischemic injury (ischemia plus edema; total lesion area minus hematoma area) (see the Figure). Volumes in cubic millimeters were calculated by multiplying the 0.5-mm slice thickness by the measured areas. To partially correct for effects of edema, all lesion areas were also expressed as percent of ipsilateral hemisphere involved. Results were analyzed with
ANOVA (unpaired 2-tailed t test). A value of $P<0.05$ was used to assess the statistical significance of differences in total lesion volume, hemorrhage volume, and ischemic injury volume between treatment groups.

**Results**

A total of 68 animals were randomized and treated. The successful placement of the needle was confirmed by the location and size of the hemorrhage as well as the animals’ exhibiting focal findings. The results of the histological volume analysis are summarized in Table 2. As expected, there was no significant difference in hematoma size between groups ($P=0.31$). Although there was a trend toward smaller total lesion size in the citicoline treatment group (24.6±12.1 mm$^3$ versus 29.8±15.2 mm$^3$), it was not significant ($P=0.09$). However, both the volume of ischemic injury and the percentage of ischemic injury were significantly smaller in the citicoline treatment group; the average total ischemic injury volume for placebo was 17.0±7.1 mm$^3$ ($t=1.6$; $P=0.048$). The total percent ischemic injury for the citicoline group was 10.8±4.3% versus 13.3±5.1% for the placebo group ($t=2.2$; $P=0.033$).

The clinical neurological focal scores and animal weights at 24 and 48 hours are summarized in Table 3. Compared with placebo, citicoline-treated animals showed improved neurological function at both time points. The average total focal score at 24 hours for the citicoline group was 11.1±2.1 (mean±SD) versus 12.8±2.5 for placebo ($t=3.0$; $P=0.003$). At 48 hours, the average total focal score for the citicoline group was 10.4±2.0 versus 12.1±2.4 for placebo ($t=3.2$; $P=0.002$). There was no significant difference between the groups in body weight over the 48-hour time period.

**Discussion**

Our study shows that treatment with citicoline in experimental intracerebral hemorrhage improves neurological functional outcome and reduces the volume of the ischemic injury surrounding the hematoma. To our knowledge, this is the first study to document both improved functional outcome and a reduction in ischemic injury volume in an experimental intracerebral hemorrhage model. It is also the first report of adapting the collagenase ICH model for mice.

The exact neuroprotective mechanism of citicoline in this study, or even in the treatment of central nervous system ischemic injury, is not known. Possible mechanisms that are supported by previous studies include a reduction in free fatty acids and free radical release, neuronal membrane stabilization, decrease in glutamate toxicity, and improved neuronal survival. Because previous studies have documented a significant ischemic area surrounding ICH, it is likely that the same neuroprotective mechanisms seen in ischemia are also operative in the current study. In our study, the color and morphological appearance of the ischemic area surrounding the hematoma on histological evaluation is identical to the area of ischemic injury that we see in a focal MCA occlusion model in the mouse. The reduction in the size of the ischemic area seen with citicoline treatment is likely a combination of an actual reduction in ischemic tissue with a decrease in edema. At 48 hours it is impossible to separate the relative amounts of ischemia and edema, and a long-term (1-month) study is needed to determine whether citicoline actually affects final infarct size. However, since much of the mortality and early morbidity in ICH occurs within 48 hours, an agent that reduces early lesion volume may have significant clinical benefit. This discussion has been added to the study.

Several prior experimental studies in the rat have shown that various pharmacological agents may produce neuroprotection and improve outcome in ICH. Lyden et al used a quantal bioassay to demonstrate that the GABA agonist muscimol was neuroprotective in a rat collagenase ICH model. In this study, varying quantities of collagenase were injected, and it was found that animals treated with muscimol were able to tolerate larger amounts of collagenase and still exhibit normal neurological function. The investigators also found that muscimol treatment helped preserve the volume of the basal ganglia and white matter at 72 hours. Rosenberg and Navratil found that treatment with the calcium channel blocker emopamil significantly reduced water content in the

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Lesion Volume, mm$^3$</th>
<th>Hemorrhage Volume, mm$^3$</th>
<th>Ischemic Injury Volume, mm$^3$</th>
<th>% Ischemic Injury Volume, mm$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo (n=34)</td>
<td>29.8±15.2</td>
<td>12.9±9.0</td>
<td>17.0±7.1</td>
<td>13.3±5.1</td>
</tr>
<tr>
<td>Citicoline 500 mg/kg (n=34)</td>
<td>24.6±12.1</td>
<td>10.8±7.4</td>
<td>13.8±5.8*</td>
<td>10.8±4.3*</td>
</tr>
</tbody>
</table>

Values are mean±SD.

* $P<0.005$ compared with placebo.
posterior region of the brain, but they did not find a reduction in edema at the hemorrhage site. Effects on neurological function were not assessed in this study. The same group also found that administration of atrial natriuretic peptide also reduced brain edema at 24 hours in a rat model of ICH. 29 Finally, Sinar et al. 30 found that pretreatment with nimodipine produced a reduction in the amount of ischemic damage and an improvement in surrounding cerebral blood flow in an autologous hemorrhagic clot model in the rat. The results of these experimental studies suggest that it is possible to reduce ICH injury through use of neuroprotective strategies.

In clinical stroke treatment trials, ICH is an exclusion for the majority of the ongoing studies. A few trials 30 do allow a small number of patients to be included if the CT scan is obtained after treatment has been initiated. Citicoline has shown promise in reducing neurological deficit in clinical ischemic stroke, 31 and further work confirming this efficacy is currently in progress. Since citicoline, unlike thrombolytics, can be administered in the field before a CT scan and has minimal side effects, it appears to be an ideal candidate for medical therapy of ICH.

Conclusions
In this experimental model of ICH, citicoline treatment significantly reduced the area of ischemic injury surrounding the hematoma and improved functional outcome at 2 days. No adverse effects of citicoline were observed in this animal model. These results support future clinical efficacy trials.

Acknowledgments
Citicoline and funds for project expenses were provided by Interneuron Pharmaceuticals, Inc. The authors would like to thank Valerie Roska for her expert assistance in the preparation of this manuscript.

References
The authors created intracerebral hemorrhage in mice by injection of bacterial collagenase. The mice were randomly treated with sodium chloride solution or citicoline, 500 mg/kg twice 24 and 48 hours after creation of the hemorrhage. Citicoline treatment was associated with an improvement in function of the mice as judged on a 28-point scale and with a significant reduction in the volume of “ischemic injury” around the hematoma. The data were suggested to support the clinical application of citicoline to patients with intracerebral hemorrhage. The experiments seem to have been conducted carefully.

I have the following comments. First, the authors report that this is the first description of the use of bacterial collagenase to induce intracerebral hemorrhage in mice. It would be useful to evaluate the model further to determine the histopathology of the hemorrhage produced and the exact nature of the surrounding “ischemia/edema.” The authors report that they measured an area of hematoma and an area of “ischemic injury” around the hematoma, as well as the combined area of both. What the ischemic area is was not determined in these studies. In a similar model in rats, the area appears to represent edema and ischemia, and it is inferred that this is the same in mice. Measurements of cerebral blood flow and metabolism would allow definitive conclusions. Drugs that have been shown to be efficacious in rats in some cases have not had such promising results in humans. The applicability to humans of effects in mice, at least from a clinical therapeutics point of view, needs to be investigated further.

Second, the magnitude of the differences between groups is very small. Although an analysis of the raw data might provide different results, as an approximation there was a statistically insignificant 16% smaller hematoma volume in the citicoline group. This percent reduction is similar to the statistically significant 19% reduction in ischemic/edema area. I wonder how much less ischemia/edema one would expect from a 16% decrease in hematoma volume independent of any possible drug effect. Further studies with different doses of collagenase might answer this question. A similar magnitude of improvement in functional score (13% to 14%) was observed. As mentioned above, drugs that have had much more marked effects in other species have had little effect in humans.

The mechanism of neuroprotection afforded by citicoline is not known. Citicoline, cytidine 5’-diphosphocholine,1 is hydrolyzed to cytidine and choline in the intestine, absorbed and resynthesized in the liver and other tissues, and then crosses the blood-brain barrier and is incorporated into membrane phospholipids. Secades and Frontera1 reported that citicoline activates membrane phospholipid synthesis, increases cerebral metabolism, restores activity of mitochondrial ATPase and membrane Na+/K+ ATPase, inhibits phospholipase A2, and increases noradrenaline and dopamine levels. Clinically, therapeutic effects have been suggested in cerebral ischemia, head injury, Alzheimer’s disease, Parkinson’s disease, and memory loss.2,3 It is conceivable that such a small effect on a hitherto undescribed pathophysiological mechanism would be of benefit. Furthermore, the drug seems to be virtually without side effects. It seems safer than some over-the-counter drugs. In view of the safety, these data by Clark et al (if confirmed by others), and those that have emerged from clinical trials in ischemic stroke, a clinical trial of citicoline in patients with intracerebral hemorrhage would seem worthwhile.2

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References
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*Stroke*. 1998;29:2136-2140
doi: 10.1161/01.STR.29.10.2136

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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