Cytidinediphosphocholine treatment to decrease traumatic brain injury–induced hippocampal neuronal death, cortical contusion volume, and neurological dysfunction in rats

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Object. In previous studies at their laboratory the authors showed that cytidinediphosphocholine (CDP-choline), an intermediate of phosphatidylcholine synthesis, decreases edema formation and blood–brain barrier disruption following traumatic brain injury (TBI). In the present study the authors investigate whether CDP-choline protects hippocampal neurons after controlled cortical impact (CCI)–induced TBI in adult rats.

Methods. After adult male Sprague–Dawley rats had been anesthetized with halothane, a moderate-grade TBI was induced with the aid of a CCI device set at a velocity of 3 m/second, creating a 2-mm deformation. Sham-operated rats, which underwent craniectomy without impact served as controls. The CDP-choline (100, 200, and 400 mg/kg body weight) or saline was injected into the animals twice (once immediately postinjury and once 6 hours postinjury). Seven days after the injury, the rats were neurologically evaluated and killed, and the number of hippocampal neurons was estimated by examining thionine-stained brain sections.

By 7 days postinjury, there was a significant amount of neuronal death in the ipsilateral hippocampus in the CA2 (by 53 ± 7%, p < 0.05) and CA3 (by 59 ± 9%, p < 0.05) regions and a contusion (volume 34 ± 8 mm³) in the ipsilateral cortex compared with sham-operated control animals. Rats subjected to TBI also displayed severe neurological deficit at 7 days postinjury. Treating rats with CDP-choline (200 and 400 mg/kg, intraperitoneally) significantly prevented TBI-induced neuronal loss in the hippocampus, decreased cortical contusion volume, and improved neurological recovery.

Conclusions. Treatment with CDP-choline decreased brain damage following TBI.

KEY WORDS • cytidinediphosphocholine • controlled cortical impact • hippocampus • neuroprotection • phospholipase • secondary neuronal death • rat

Cytidinediphosphocholine (CDP-choline or citicoline) is an intermediate in the synthesis of PC, which is essential for membrane integrity and repair. Recent studies have shown that administration of CDP-choline decreases the cognitive impairment, BBB breakdown, and edema formation observed after CCI-induced TBI in adult rats. A CCI injury leads to a significant amount of secondary neuronal death in the hippocampal CA2 and CA3 regions, a focal necrotic cavitation in the injured cortex, and neurological dysfunction by 7 days after the injury. In the present study we evaluated whether CDP-choline treatment decreases hippocampal neuronal loss, cortical contusion, and neurological dysfunction following CCI injury.

Abbreviations used in this paper: ANOVA = analysis of variance; BBB = blood–brain barrier; CCI = controlled cortical impact; CDP-choline = cytidinediphosphocholine; FP = fluid percussion; MABP = mean arterial blood pressure; NIH = National Institutes of Health; NMDA = N-methyl-D-aspartate; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PLA2 = phospholipase A2; PS = phosphatidylserine; SD = standard deviation; TBI = traumatic brain injury.

Materials and Methods

Traumatic Brain Injury

Controlled cortical impact injury was induced in 32 adult male Sprague–Dawley rats, each weighing between 250 and 280 g, as described earlier. After the animals had been anesthetized with halothane, a craniectomy (6 mm in diameter, extending between the bregma and lambda, 1 mm lateral to the midline) was performed and a moderate grade of injury was delivered at a velocity of 3 m/second, resulting in a 2-mm deformation. The exposed cortex was covered with Surgicel and the wound was closed with sutures. A group of sham-operated rats underwent craniectomy without impact. All surgical procedures and animal care were conducted according to animal-welfare guidelines (1985 Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23) and were approved by the animal care committee of the University of Wisconsin–Madison. After the rats recovered from anesthesia, they were returned to their cages and given free access to rat chow and water. During surgery, the animals’ body and cranial temperatures were monitored using rectal and temporalis muscle probes and maintained at 37 to 38°C (rectal) and 36 to 37°C (cranial). The left femoral artery was cannulated for continuous monitoring of MABP and arterial blood gas levels after induction and maintenance of anesthesia, as described previously. Physiological parameters were monitored up to 60 minutes after the injury.
Drug Treatment

Rats subjected to CCI injury were divided into four groups with eight animals in each group. Intraperitoneal injections of CDP-choline (0.5 ml) or saline were administered to the animals. Each rat received two injections—one immediately after the injury (≤3 minutes postinjury) and the other 6 hours later. Rats in Groups 1, 2, and 3 received CDP-choline at concentrations of 100, 200, and 400 mg/kg body weight, respectively, and rats in Group 4 received saline. The sham-operated animals received either saline (eight animals) or CDP-choline (400 mg/kg body weight; eight animals). These doses were chosen based on our previous studies of CDP-choline after TBI and cerebral ischemia.4,30

Histopathological Study

Estimates were made of CCI injury–induced hippocampal neuronal loss and cortical contusion volume in a manner described earlier.10,14 All rats were killed 7 days postinjury. Each brain was sectioned coronally (40-μm-thick slices at an interval of 320 μm; from −2.4 mm to −6.5 mm with respect to the bregma, covering the dorsal hippocampus according to the rat brain atlas of Paxinos and Watson19,33) and the sections were stained with thionine. Live neurons were counted in the hippocampal regions of CA1, CA2, and CA3 by an evaluator blinded to the study groups. For each animal, mean counts of neurons were obtained by examining at least five serial coronal sections in the dorsal hippocampus underlying the area of contusion. In each section, the neurons were counted in three nonoverlapping fields at a magnification of × 400 in each subregion (CA1, CA2, and CA3). This strategy significantly improved the estimate of the number of hippocampal neurons by eliminating nonrepresentative sampling and geometric biases. Only complete neuronal cells with a clearly defined cell body and nucleus were counted. The cortical contusion volume was computed for each brain as described earlier.4,34 For this, 12 sections covering the area of injury (−2.4 mm to −6.5 mm with respect to the bregma) were imaged on a flat-bed scanner and the contusion volume was measured using the public domain NIH Image J (version 1.29) program.

Neurological Evaluation

Neurological deficits were evaluated 1 day before TBI and on Days 1, 4, and 7 after TBI by an investigator blinded to the study groups. A highly consistent neurological scoring system (with a 90% interobserver reliability) that can discriminate the level of injury and the effects of drugs after TBI in rats was used.10,34 The neurological scores reflect a composite score of a battery of tests including resistance to forced lateral pulsion (on both right and left sides), forelimb contralaxion (both right and left sides) while the animal is suspended by its tail, and the ability to maintain position on a vertical inclined plane. Scores range from 0 (maximum deficit) to 5 (normal function) for each task and the composite neurological scores range from 0 to 25.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham-Operated/ Saline-Treated</th>
<th>Sham-Operated/ CDP-Choline–Treated</th>
<th>TBI/Saline-Treated</th>
<th>TBI/CDP-Choline–Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP (mm Hg)</td>
<td>94.8 ± 7.8 90.1 ± 8.2</td>
<td>95.2 ± 6.5 89.2 ± 7.3</td>
<td>93.6 ± 8.0 88.8 ± 6.9</td>
<td>91.7 ± 8.2 88.2 ± 7.3</td>
</tr>
<tr>
<td>PO₂ (mm Hg)</td>
<td>143.6 ± 34.5 139.3 ± 36.1</td>
<td>151.1 ± 40.4 153.2 ± 32.1</td>
<td>138.1 ± 41.1 142.2 ± 29.7</td>
<td>149.9 ± 38.9 154.5 ± 45.6</td>
</tr>
<tr>
<td>PCO₂ (mm Hg)</td>
<td>39.6 ± 3.2 44.3 ± 5.1</td>
<td>42.4 ± 4.8 38.9 ± 5.6</td>
<td>37.7 ± 4.9 41.5 ± 4.7</td>
<td>45.8 ± 6.2 41.6 ± 5.7</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 ± 0.02 7.33 ± 0.03</td>
<td>7.32 ± 0.03 7.34 ± 0.03</td>
<td>7.34 ± 0.04 7.37 ± 0.04</td>
<td>7.38 ± 0.03 7.33 ± 0.03</td>
</tr>
<tr>
<td>hematocrit (%)</td>
<td>35.8 ± 3.7 33.6 ± 5.1</td>
<td>34.4 ± 4.6 37.4 ± 4.2</td>
<td>35.5 ± 4.2 34.3 ± 2.9</td>
<td>34.4 ± 3.7 33.2 ± 4.1</td>
</tr>
<tr>
<td>brain temp (°C)†</td>
<td>36.5 ± 0.3 36.8 ± 0.4</td>
<td>36.3 ± 0.4 36.7 ± 0.3</td>
<td>36.5 ± 0.4 36.7 ± 0.2</td>
<td>36.7 ± 0.4 36.8 ± 0.3</td>
</tr>
<tr>
<td>body temp (°C)‡</td>
<td>37.5 ± 0.3 37.3 ± 0.3</td>
<td>37.8 ± 0.4 37.6 ± 0.3</td>
<td>37.4 ± 0.3 37.9 ± 0.5</td>
<td>37.5 ± 0.4 37.8 ± 0.3</td>
</tr>
</tbody>
</table>

* All physiological variables were measured 5 minutes before (preop) and 60 minutes after (postop) TBI or sham surgery. Each value is expressed as the mean ± SD. Data for the 400-mg/kg body weight CDP-choline groups are displayed. The other two doses (100 and 200 mg/kg) also caused no significant changes in any parameter analyzed.

† Measured rectally.
‡ Measured in temporalis muscle.

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TABLE 1
Physiological parameters in rats injected with saline or CDP-choline after a sham operation or CCI injury*

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
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</thead>
<tbody>
<tr>
<td>sham-operated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline</td>
<td>84 ± 13</td>
<td>79 ± 10</td>
<td>110 ± 15</td>
</tr>
<tr>
<td>CDP-C (400 mg/kg)</td>
<td>80 ± 11</td>
<td>84 ± 13</td>
<td>105 ± 14</td>
</tr>
<tr>
<td>TBI saline</td>
<td>72 ± 14</td>
<td>68 ± 13</td>
<td>99 ± 12</td>
</tr>
<tr>
<td>CDP-C (100 mg/kg)</td>
<td>69 ± 10</td>
<td>74 ± 11</td>
<td>101 ± 11</td>
</tr>
<tr>
<td>CDP-C (200 mg/kg)</td>
<td>76 ± 12</td>
<td>69 ± 14</td>
<td>104 ± 16</td>
</tr>
<tr>
<td>CDP-C (400 mg/kg)</td>
<td>78 ± 14</td>
<td>75 ± 13</td>
<td>97 ± 14</td>
</tr>
</tbody>
</table>

* Each value is expressed as the mean ± SD of eight rats/group. Statistical analysis was performed using a one-way ANOVA followed by the Tukey–Kramer multiple-comparisons posttest. Abbreviation: CDP-C = CDP-choline.
† p < 0.05 compared with the respective sham-operated control group (one-way ANOVA followed by the Bonferroni t-test).
‡ p < 0.05 compared with the saline-injected TBI group.

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Results

Treatment with CDP-choline did not alter the physiological parameters. There were no statistically significant differences between groups injected with saline and those injected with CDP-choline in any physiological parameter (pH, PCO₂, PO₂, hemoglobin, blood glucose, and temporals muscle and rectal temperatures) or MABP monitored during TBI (Table 1). There was no death in saline- or CDP-choline–injected rats with TBI.

Treatment with CDP-choline decreased the TBI-induced death of hippocampal neurons. In saline-injected rats, CCI injury caused significant neuronal loss after 7 days in the CA2 (by 53%, \( p < 0.05 \)) and CA3 (by 59%, \( p < 0.05 \)) regions of the hippocampus, compared with sham-operated control animals (Fig. 1, Table 2). Treating rats with CDP-choline at a dose of 100 mg/kg had no effect on the TBI-induced death of neurons (Table 2), whereas 200 mg/kg CDP-choline significantly prevented this neuronal death in both the CA2 (by 76%, \( p < 0.05 \)) and CA3 (by 88%, \( p < 0.05 \)) regions compared with saline-injected control animals (Fig. 1, Table 2). Increasing the dose of CDP-choline to 400 mg/kg had no further protective effect against TBI-induced neuronal death (Table 2). Injecting sham-operated rats with CDP-choline resulted in no significant change in the number of live neurons in the CA1, CA2, and CA3 regions, compared with saline-injected control animals (Table 2).

The volume of cortical contusion was significantly decreased by CDP-choline. Similar to previous studies, a moderate-grade CCI injury led to a focal necrotic cavitation in the ipsilateral cortex by 7 days postinjury. Treatment with CDP-choline (200 or 400 mg/kg) significantly reduced the posttraumatic contusion volume by 41% (from 34 ± 8 mm³ in saline-treated rats to 20 ± 5 mm³ in CDP-choline–treated rats, \( p < 0.05 \); Fig. 2).

Treatment with CDP-choline improved neurological recovery in rats after TBI. In saline-treated rats, CCI injury led to severe impairment of neurological function at Day 1 with a significant decrease (\( p < 0.05 \)) in the median neurological score to 7 from a preinjury score of 25; the score recovered to 15 by Day 4 and to 16 by Day 7 (Table 3). This is similar to previously published observations.

In rats treated with CDP-choline (200 or 400 mg/kg) after CCI injury, the median neurological score decreased to 6 at Day 1, but recovered to 14 by Day 4 and to 22 by Day 7 (Table 3). This indicates an improved capability for neurological recovery in CDP-choline–treated rats. There were no significant differences between sham-operated rats injected with saline or CDP-choline in their median neurological scores on Days 1, 4, or 7 (Table 3).

Discussion

Treatment with CDP-choline prevents the excitotoxic death of neurons. The present study shows that CDP-choline treatment prevents the death of hippocampal neurons and decreases cortical contusion and neurological dysfunction following moderate TBI. Previous studies from our laboratory and other groups also have shown that CDP-choline decreases posttraumatic edema formation, BBB disruption, and cognitive impairment. Both CCI and FP models can produce graded, reproducible, and quantifiable brain injury in rats. The cortical injury, loss of hippocampal neurons, and neurological deficits produced by a moderate-grade CCI injury (2-mm deformation caused by the device set at a 3-m/second velocity) are comparable to those produced by a moderate-grade FP injury (induced by applying a 2-atm pressure pulse).

Authors of previous studies have defined the severity of the CCI injury in rats by studying the pathological effects of varying compression and impact velocity. Using a 3-m/second velocity, these researchers have graded CCI injuries as mild (1-mm deformation), moderate (2-mm deformation), and severe (3-mm deformation). In the present study we created a 2-mm deformation by applying a 3-m/second velocity, which corresponds to a moderate grade of injury. We restricted our studies to moderate injury because severe CCI causes inconsistent and extensive brain damage with a high rate of mortality. Hence, drug trials in cases of severe injury often lead to irreproducible data. The effectiveness of CDP-choline after severe TBI is not known. The neuroprotection afforded by CDP-choline after TBI is apparently not the result of systemic alterations, as the physiological parameters were observed to be identical between CDP-choline– and saline-treated rats. Several previous studies have also demonstrated promising neuroprotection by CDP-choline in animal models of transient cerebral ischemia and subarachnoid hemorrhage.
Treatment with CDP-choline improves neurological recovery after TBI. Previous studies have demonstrated that moderate TBI induced by either CCI or FP in adult rats leads to acute neurological deficits that typically include a loss of resistance to a lateral push; minimal extension of the forelimb, leading to an inability to prevent the nose from touching the ground after a forelimb contraflexion; and an inability to maintain position on an inclined plane. Treatment with CDP-choline significantly prevented CCI injury–induced neuronal death in the ipsilateral CA2 and CA3 regions compared with saline treatment. The mean ± SD numbers of neurons in the contralateral and ipsilateral CA1, CA2, and CA3 regions in each group (eight animals/group) are given in Table 2. Original magnification × 40 (upper and center panels) and × 100 (lower panels).

![Photomicrographs of thionine-stained brain sections showing the contralateral (Contra) and ipsilateral (Ipsi) hippocampus of rats subjected to CCI injury, which were injected with saline or 400 mg/kg CDP-choline. In the saline-injected group the CCI injury caused significant neuronal loss in the CA2 and CA3 regions of the ipsilateral hippocampus when compared with the contralateral hippocampus. Treatment with CDP-choline significantly prevented CCI injury–induced neuronal death in the ipsilateral CA2 and CA3 regions compared with saline treatment. The mean ± SD numbers of neurons in the contralateral and ipsilateral CA1, CA2, and CA3 regions in each group (eight animals/group) are given in Table 2. Original magnification × 40 (upper and center panels) and × 100 (lower panels).](image-url)

Calcium-dependent processes downstream to NMDA receptors promote posttraumatic neuronal death. One possible mechanism for the neuroprotection provided by CDP-choline involves its effect on the biochemical pathways stimulated during posttraumatic excitotoxicity. Although...
the precise molecular mechanism responsible for secondary hippocampal neuronal death following TBI is not yet known, glutamate-mediated excitotoxicity is thought to be the starting point. Following a traumatic insult, due to an increased release and decreased reuptake of glutamate, concentrations of this substance rise in the synaptic cleft, resulting in ionotropic and metabotropic overstimulation of the glutamate receptor. Of the various subtypes of glutamate receptors, stimulation of the NMDA subtype is the main precipitator of posttraumatic neuronal death. Although several NMDA receptor blockers (memantine, ifenprodil, phencyclidine, MK801, HU-211, ketamine, dextrorphan, and remacemide) decrease TBI-induced brain damage in experimental animal models, none has conclusively shown neuroprotection clinically. The majority of human trials in which NMDA antagonists were administered have been discontinued because of undesirable psychomimetic effects. A more focused approach to minimize the trauma-induced death of neurons is to prevent processes downstream to NMDA receptors. Activation of the NMDA receptor after TBI leads to a massive influx of calcium ions, which stimulate several potentially neurotoxic calcium-dependent enzymes such as calpain, nitric oxide synthase, and PLA2. The results of previous studies have shown that calpain and nitric oxide synthase inhibitors decrease the trauma-induced death of neurons. Prevention of PLA2 activation is central to the neuroprotection afforded by CDP-choline. Phosphatidylethanolamine, PS, PI, PC, and sphingomyelin are the five phospholipids that constitute major components of the plasma membrane. Under normal conditions, the anionic phospholipids (PE, PS, and PI) are present in the cytofacial leaflet and the neutral phospholipids (PC and sphingomyelin) are present in the exofacial leaflet of the plasma membrane. This asymmetrical distribution of phospholipids is essential for plasma membrane integrity. Excitotoxic insults lead to translocation of phospholipids between the cyto- and exofacial leaflets, leading to increased membrane fluidity and membrane damage. As PLA2 hydrolyzes the membrane phospholipids PC and PE, overactivation of PLA2 following TBI leads to the loss of these two phospholipids and damage to the membrane. Hydrolysis of PC and PE releases the free fatty acid arachidonic acid. The released arachidonic acid is metabolized to form leukotrienes, prostaglandins, and reactive oxygen species, which promote lipid peroxidation, edema, and neuronal death. Recent studies have demonstrated that CDP-choline restores the concentrations of PC following cerebral ischemia by increasing synthesis of PC from diacylglycerol and by preventing activation of PLA2. Prevention of PLA2 activation leads to decreased amounts of arachidonic acid and reactive oxygen species, which prevent lipid peroxidation, edema, and neuronal damage. Reactive oxygen species also promote glutathione depletion, leading to oxidative damage in the post-traumatic brain, and the choline moiety of CDP-choline can be used to restore glutathione levels to decrease the oxidative stress. The putative neuroprotective mechanisms of CDP-choline are shown in Fig. 3.

In combination with other drugs CDP-choline promotes synergistic neuroprotection. As multiple molecular mechanisms promote posttraumatic neuronal death synergistically, a combined therapy with drugs that act on different neurotoxic pathways might promote improved protection.
Recent studies have shown that CDP-choline, when used in combination with urokinase, recombinant tissue plasminogen activator, basic fibroblast growth factor, or MK801, exhibits a synergistic neuroprotective effect in cerebral ischemia models. Future studies will address the possibility of using CDP-choline in combination with other agents to prevent the neuronal damage that occurs after TBI. Furthermore, the efficacy of CDP-choline was not tested in other experimental TBI models. To date, CDP-choline has not been used in a clinical trial in patients with TBI.

Conclusions

Treatment with CDP-choline significantly prevented TBI-induced secondary neuronal death in the rat hippocampus. The neurological recovery observed after TBI was significantly enhanced in CDP-choline–treated rats. Taken together with the results of previous studies, CDP-choline has the potential to decrease edema, BBB disruption, and death of neurons after TBI. Compared with traditional NMDA receptor blockers, CDP-choline is nontoxic and it may prove to be useful as one of a combination of agents in clinical studies.

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