Neuroprotection by the stable nitroxide 3-carbamoyl-proxyl during reperfusion in a rat model of transient focal ischemia

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OBJECT. Nitroxides mimic superoxide dismutase (SOD) biochemically and may prevent free radical oxidative injury in settings in which endogenous SOD is overwhelmed. The authors have previously shown the efficacy of a nitroxide, Tempol, in reducing stroke infarct size. Of the nitroxides, 3-carbamoyl-proxyl (3-CP) is especially promising for clinical use, because it does not cause hypotenension in animals. Its efficacy in brain ischemia, however, is untested. The goal of this study was to ascertain whether 3-CP would reduce brain damage in a rat ischemia–reperfusion model.

METHODS. The authors performed a blinded, dose–response study of the effect of different amounts of 3-CP (1, 10, and 100 mg/kg) on infarct size at 24 hours after focal ischemia and reperfusion. The 3-CP was given intravenously during reperfusion, which followed 1 hour of reversible ischemia induced by a thread placed intraluminally in the middle cerebral artery of rats. Brain infarcts, measured with 2,3,5-triphenyltetrazolium chloride staining in six 3-CP groups, were compared with those measured in controls (animals given an equal volume of saline).

Edema-corrected infarct sizes (mean ± standard deviation) were as follows: 146 ± 64 mm$^3$ in controls; 107 ± 18 mm$^3$ in rats given 1 mg/kg 3-CP; 40 ± 20 mm$^3$ in those given 10 mg/kg 3-CP; and 44 ± 17 mm$^3$ in those given 100 mg/kg 3-CP. A statistically significant reduction in infarct size was achieved in the 10- and 100-mg/kg 3-CP–treated groups (p < 0.01). A reduction in infarct size was also seen in the 1 mg/kg 3-CP–treated group, but this did not reach statistical significance. The authors observed no effects of 3-CP on blood pressure or brain temperature.

CONCLUSIONS. Given at reperfusion, 3-CP significantly decreases brain infarct size at doses of 10 and 100 mg/kg without causing hypotension. The authors found that 3-CP is well suited for further laboratory and clinical use in brain ischemia and reperfusion.

KEY WORDS • cerebral ischemia • reperfusion damage • 3-carbamoyl-proxyl • nitroxide • rat

S T A B L E Nitroxides are a promising group of compounds for use in cerebral ischemia and protection against its effects, because they mimic an extremely important endogenous free radical scavenger, SOD.19,25 In addition to their direct catalytic role as an SOD mimic, nitroxides can act as procatalysts by stimulating catalase-like activities in heme proteins11 and can provide antioxidant defense by detoxifying oxidants such as hydrogen peroxide and organic peroxides.19,26 We have previously reported on the effectiveness of the nitroxide Tempol in a model of focal rat brain ischemia.24 Tempol was studied because of its low toxicity and ability to cross the blood–brain barrier. Although it is effective for reduction of infarct size at doses that do not cause significant hypotension in rat, porcine,9 and murine8 models, high-dose Tempol causes hypotension and reflex tachycardia due to systemic vasodilation. A related nitroxide, 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidinyl-N-oxyl, or 3-CP, did not cause hypotension in miniature pigs8 or in mice8 and may be more suitable for clinical use because hypotension combined with cerebral ischemia is undesirable. To test the potential benefit of 3-CP in the treatment of brain ischemia, we performed a dose–response study of the effects of 3-CP on stroke in rats at 24 hours after focal ischemia and reperfusion.

MATERIALS AND METHODS

Animal Preparation

Male Sprague–Dawley rats, each weighing between 280 and 350 g, were used in accordance with an institutional review board protocol. The animals were provided with unlimited access to food and water. The 3-CP was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in isotonic saline immediately before each experiment. The effects of 3-CP during brain reperfusion after ischemia were investigated in four different groups. Animals were randomized as follows (six rats in each group): 100 mg/kg 3-CP; 10 mg/kg 3-CP; 1 mg/kg 3-CP; and vehicle (0.9% saline) groups. Animals were initially anesthetized with 4% isoflurane. After tracheal intubation, anesthesia was maintained with 2% isoflurane in a carrier gas of N$_2$/O$_2$ (2:1). The rats received mechanical ventilation with a rodent volume ventilator (model 683; Harvard Apparatus, Holliston, MA) adjusted to a rate that maintained normocarbia according to arterial blood gas sampling (I-STAT Clinical Analyzer; Heska Corp., Fort Collins, CO). Each animal’s rectal temperature was monitored to maintain normothermia (36.5–37.5°C) with a thermostatically regulated heating pad (CMA model 150; CMA/Microdialysis AB, Solna, Sweden). A polyethylene catheter was introduced into the femoral artery for continuous monitoring of blood pressure. Physiological variables were recorded continuously on a personal computer using Polyview Data Acquisition 2.0 software (Astro-Med, Inc., West Warwick, RI) and tabulated at six representative time points throughout the experiment for intergroup comparisons. The right
Fig. 1. Bar graph showing infarct volume after correction for edema. The error bars represent the mean ± SD; the asterisk indicates statistical significance. A reduction in the infarct size is seen with 1 mg/kg of 3-CP, but does not reach statistical significance.

Occlusion of the MCA

Transient MCA occlusion was performed in all groups by using an intraluminal thread technique, as follows. The neck was opened with a ventral midline skin incision. With the aid of an operating microscope, the right common carotid artery and its ramifications were exposed. The pterygopalatine artery was ligated at the ICA bifurcation, the right common carotid artery and its ramifications were exposed. The pterygopalatine artery was ligated at the ICA bifurcation, and the external carotid artery was divided distally and used as a conduit to access the ICA. After placing a microvacular clip across the common carotid artery, a 25-mm length of a 4.0 monofilament, silicone-coated monofilament suture was introduced into the ICA lumen. The suture was then advanced slowly and gently until the rCBF dropped (typically to 25% of baseline), as measured using a laser Doppler perfusion probe. After a 60-minute occlusion, the thread was withdrawn, and reperfusion was confirmed with the laser Doppler probe. Either failure to achieve an appropriate drop in rCBF or failure to achieve reperfusion was an exclusion criterion.

Animals were extubated after the return of spontaneous breathing and recovered on a heating pad until fully active.

Drug Administration

In all groups, the investigators were blinded to whether drug or vehicle had been administered. The 3-CP solutions in 1 ml of 0.1 M phosphate-buffered saline (pH 7.4) or 1 ml vehicle (phosphate-buffered saline) were infused over 1 minute immediately after the onset of reperfusion.

 Determination of Infarct Size

After 24 hours of reperfusion, we gave the rats a lethal dose of pentobarbital. Their brains were immediately removed and placed in a graduated matrix for tissue slicing. Two-millimeter coronal sections were cut and stained in 2% 3,3,5-triphenyltetrazolium chloride for 20 minutes at 37˚C, and the slices were then fixed and stored in 10% formalin. We digitally photographed stained sections and outlined the area of infarction (nonred 2,3,5-triphenyltetrazolium chloride-stained tissue) by using a computer image analysis system (Image Pro Plus version 4.1; Media Cybernetics, Silver Spring, MD). Infarct volumes, in cubic millimeters, were calculated using Cavalieri’s principle.15 The presence of cerebral edema was corrected for by dividing the total infarct volume by the ratio of the total right hemisphere/left hemisphere volume. This method assumes equal-sized hemispheres before ischemia and the absence of significant edema in the left hemisphere.

TABLE 1
Physiological variables in rats given 3-CP after transient focal ischemia*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline Value</th>
<th>5 Mins</th>
<th>30 Mins</th>
<th>60 Mins</th>
<th>10 Mins</th>
<th>30 Mins</th>
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<tr>
<td>MABP (mm Hg)</td>
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<td></td>
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<tr>
<td>vehicle</td>
<td>106.0 ± 9.0</td>
<td>99.0 ± 9.0</td>
<td>106.5 ± 12.5</td>
<td>108.0 ± 15.8</td>
<td>112.3 ± 8.3</td>
<td>108.3 ± 8.4</td>
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<td>3-CP</td>
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<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>102.1 ± 3.7</td>
<td>98.8 ± 1.4</td>
<td>100.8 ± 3.8</td>
<td>99.2 ± 2.6</td>
<td>104.2 ± 8.0</td>
<td>101.7 ± 4.4</td>
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<td>10 mg/kg</td>
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<td>97.5 ± 3.2</td>
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<td>100.4 ± 6.6</td>
<td>98.3 ± 11.4</td>
<td>102.9 ± 11.9</td>
<td>99.2 ± 6.5</td>
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<tr>
<td>vehicle</td>
<td>37.1 ± 0.3</td>
<td>37.4 ± 0.2</td>
<td>37.0 ± 0.5</td>
<td>37.2 ± 0.4</td>
<td>37.4 ± 0.2</td>
<td>37.3 ± 0.3</td>
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<td>3-CP</td>
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<tr>
<td>1 mg/kg</td>
<td>37.2 ± 0.3</td>
<td>37.1 ± 0.2</td>
<td>37.1 ± 0.2</td>
<td>37.1 ± 0.2</td>
<td>37.4 ± 0.2</td>
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<tr>
<td>10 mg/kg</td>
<td>37.1 ± 0.2</td>
<td>37.1 ± 0.2</td>
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<tr>
<td>100 mg/kg</td>
<td>37.1 ± 0.2</td>
<td>37.1 ± 0.3</td>
<td>37.2 ± 0.3</td>
<td>37.1 ± 0.3</td>
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<td>37.2 ± 0.3</td>
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<tr>
<td>cranial temp (˚C)</td>
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<td>vehicle</td>
<td>36.3 ± 0.8</td>
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<td>36.3 ± 0.8</td>
<td>36.5 ± 1.1</td>
<td>36.3 ± 0.9</td>
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<tr>
<td>1 mg/kg</td>
<td>36.6 ± 0.6</td>
<td>36.5 ± 0.6</td>
<td>36.5 ± 0.5</td>
<td>36.5 ± 0.5</td>
<td>36.7 ± 0.4</td>
<td>37.7 ± 0.4</td>
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<tr>
<td>10 mg/kg</td>
<td>36.9 ± 0.4</td>
<td>37.0 ± 0.3</td>
<td>36.9 ± 0.3</td>
<td>36.9 ± 0.3</td>
<td>37.0 ± 0.4</td>
<td>37.0 ± 0.4</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>36.9 ± 0.4</td>
<td>36.8 ± 0.3</td>
<td>36.9 ± 0.4</td>
<td>36.8 ± 0.2</td>
<td>36.8 ± 0.5</td>
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<td>rCBF (% of baseline)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>vehicle</td>
<td>100</td>
<td>14.5 ± 6.9</td>
<td>18.6 ± 9.1</td>
<td>23.0 ± 12.8</td>
<td>104.4 ± 8.8</td>
<td>112.8 ± 29.6</td>
</tr>
<tr>
<td>3-CP</td>
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<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>100</td>
<td>19.0 ± 17.3</td>
<td>22.6 ± 16.6</td>
<td>26.0 ± 14.2</td>
<td>132.2 ± 40.9</td>
<td>117.4 ± 20.1</td>
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<td>10 mg/kg</td>
<td>100</td>
<td>20.9 ± 6.4</td>
<td>20.8 ± 6.1</td>
<td>21.0 ± 5.4</td>
<td>115.0 ± 32.5</td>
<td>112.9 ± 37.1</td>
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<tr>
<td>100 mg/kg</td>
<td>100</td>
<td>22.0 ± 8.0</td>
<td>24.1 ± 4.9</td>
<td>24.4 ± 5.0</td>
<td>108.3 ± 6.2</td>
<td>106.4 ± 9.0</td>
</tr>
</tbody>
</table>

* Values are expressed as the means ± SDs. A statistical analysis of physiological variables at defined time points during the experiment failed to reveal significant differences between groups. Abbreviation: temp = temperature.
Nitroxide 3-carbamoyl-proxyl for brain protection

Results

Seven animals were excluded before randomization because of failure to meet the inclusion criteria for the ischemia model. One animal in the vehicle group died before histopathological studies were performed and thus the data were analyzed using a sample size of five for the control and six for the other groups.

Edema-corrected infarct sizes in the four groups were as follows (mean ± SD); 146 ± 64 mm³ in controls; 107 ± 18 mm³ in the 1-mg/kg 3-CP group; 40 ± 20 mm³ in the 10-mg/kg 3-CP group; and 44 ± 17 mm³ in the 100-mg/kg 3-CP group (Fig. 1). One-way ANOVA revealed a significant drug treatment effect on mean infarct volume (F(3, 19) = 12.98, p < 0.0001). A post hoc comparison analysis indicated that the 10- and 100-mg/kg 3-CP treatment groups had significantly reduced infarct volumes compared with control animals (p < 0.01). The statistical significance was essentially identical with and without edema correction.

A comparison of physiological variables between 3-CP–treated animals and controls yielded no significant differences between groups as far as values for MABP, rectal temperature, cranial temperature, and rCBF. The 3-CP did not affect rCBF during reperfusion (Table 1). Lack of blood pressure effects of 3-CP compared with controls is demonstrated graphically in Fig. 2. A comparison of arterial blood gas variables between 3-CP–treated animals and control rats also yielded no significant differences between groups in values for pH, PCO₂, and PO₂ (Table 2).

Discussion

Given at reperfusion, 3-CP caused a significant, dose-dependent reduction in brain infarct size in rats 24 hours after ischemia and reperfusion. We observed no deleterious effects of its use, even at a dosage of 100 mg/kg. The infarct reduction was comparable to that seen with use of its related compound, Tempol.²⁴

The group of chemicals to which 3-CP belongs, the stable nitroxides, have been demonstrated to be potent antioxidants.¹⁰,¹⁹ Biochemical SOD-like activity in these substances has been shown in vitro.²² The in vivo effects of nitroxides include reduction of radiation-induced cell damage and death in experimental animals.⁸,¹⁶–¹⁸ Topical application can prevent radiation-related alopecia in rodents.⁴ Myocardial reperfusion damage can be prevented with the nitroxide Tempol if it is given just before reperfusion.³ Cerebral infarction may also be ameliorated by nitroxides in rats and by nitrones in nonhuman primates,¹²,²⁴ but data are lacking in humans.

Cerebral reperfusion after embolic stroke is the goal of “brain attack” treatment with thrombolysis. Reperfusion of ischemic tissue, however, results in the production of reactive oxygen species that can paradoxically damage and kill tissue. In rabbits given thrombolysis after embolic stroke, reactive oxygen species generation increases compared with animals that remain untreated.²³ Although SOD is an endogenous scavenger that protects tissue from reperfusion injury, its ability to do so can be overwhelmed. There is some evidence that this is relevant to the pathophysiological features of human stroke, because SOD activity is diminished in human blood after cerebral infarction.²⁷ Supplementing endogenous SOD activity, without causing hypotension, was our objective in using 3-CP. The reduction in infarct size following treatment with this agent compares favorably with that seen with use of its related compound, Tempol, in this model.²⁴ Based on the experience with animals, we believe that 3-CP may be more suit-
able for clinical trials than Tempol, because the latter causes systemic vasodilation and hypotension at high doses.

At a dose of 100 mg/kg, 3-CP did not enhance histological protection compared with a dose of 10 mg/kg; both amounts yielded an approximately 70% reduction in infarct size (Fig. 1). No agent given after the onset of ischemia (in this case after 1 hour of ischemia) can be expected to prevent all histological damage, but the clinical relevance is greater in a postictal treatment model than in a prophylactic one. The goal of our study design was to salvage the ischemic penumbra. This smaller area of infarction represents the core of ischemia and the limit of our ability to protect the brain after ischemia; thus the absence of increased histological protection of a 100-mg over a 10-mg dose is not surprising.

To date, there has been great disappointment in the results of clinical use of pharmacological agents for neuroprotection. This includes a recent flurry of negative Type I data in studies of the effects of these drugs on stroke and head injury from randomized prospective Phase II and III trials,1–3,5,12,20,21 in which agents that have been proven effective in animal models, such as lazeroids, N-methyl-D-aspartate antagonists, and sodium channel antagonists, are being tested. Because oxygen free radical species generation is an important component of ischemic injury14,23 and nitroxide supplementation of endogenous SOD activity has not been clinically tested, we believe that 3-CP has the potential for clinical use, based on data obtained in these animals.

Disclaimer
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References

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