Carnitine Treatment for Stroke in Rats

Andrew Slivka, MD, David Silbersweig, MD, and William Pulsinelli, MD, PhD

Changes in the concentrations of carnitine, long-chain acylcoenzyme A, and long-chain acylcarnitine in ischemic myocardium parallel those in ischemic brain. Since carnitine treatment reverses these changes and improves function in ischemic hearts, we examined whether carnitine given to rats before focal cerebral ischemia (produced by tandem right common carotid artery and middle cerebral artery occlusion) alters infarct volume in four separate experiments. Mannitol was used to control for the osmotic effect of carnitine on brain edema in one experiment. While carnitine was found to significantly decrease infarct volume compared with saline in one experiment \((p<0.05, \text{Student's} \ t \ \text{test})\), this result could not be replicated in the subsequent three experiments. Because the positive treatment effect was not reproducible despite similar experimental conditions, the result of the first experiment was attributed to a type I error. Mannitol also showed no significant effect on infarct volume. This study emphasizes the need for concurrent controls with each group of treated animals and the need for replicating the results of a single experiment when testing for drug efficacy in animal models of focal cerebral ischemia. (Stroke 1990;21:808-811)

Carnitine (3-hydroxy-4-\(N\)-trimethylammoniumbutyrate), which functions as the mitochondrial carrier for long-chain acylcoenzyme A (acyl-CoA), has shown promise in the treatment of myocardial ischemia. Carnitine stores are depleted with myocardial ischemia, leading to the inability to oxidize long-chain fatty acids and an increased synthesis of triglycerides, which result in the accumulation of long-chain acyl-CoA and long-chain acylcarnitine esters.1-4 Elevated levels of tissue acyl-CoA further impair energy metabolism by inhibiting adenine nucleotide translocase activity and decreasing adenosine triphosphate production.4,5 Also, long-chain acylcarnitine as well as free fatty acids may damage membrane structures by their detergent actions.6

Fifteen minutes of global cerebral ischemia in dogs causes a 50% decline in the concentration of free carnitine and a corresponding rise in the levels of acylcarnitine.7 Transient forebrain ischemia in both dogs and rats results in increased tissue levels of long-chain acyl-CoA that roughly parallel the severity of ischemia as measured by tissue lactate concentrations.8 Increasing carnitine levels in the brain would theoretically reverse these changes and their potential deleterious consequences, as has been shown in myocardial ischemia.8-11 Therefore, we examined whether carnitine given to rats before focal cerebral ischemia altered the volume of cerebral infarcts.

**Materials and Methods**

One hundred nine fasted male spontaneously hypertensive rats weighing 210–310 g were anesthetized with 1.5–2.5% halothane. The tail artery was cannulated with a polyethylene catheter (PE-50) to monitor blood pressure and to obtain blood samples for physiologic variables. Focal neocortical ischemia was produced according to the method described by Brint et al.12 Briefly, the right common carotid artery (CCA) was occluded with 4-0 surgical silk. The right middle cerebral artery (MCA) was exposed through a 2-mm burr hole drilled under a continuous normal saline drip several millimeters rostral to the fusion of the zygomatic arch with the squamosal bone. Using a MM 3 micromanipulator (Narishige Instruments, Tokyo, Japan), a hook formed of 20-gauge silver wire was positioned under the MCA. The MCA was then lifted 0.5-1 mm above the cortical surface and cauterized. Body temperature was maintained at 37°C throughout the procedure with a heat lamp connected to a rectal thermistor. Immediately after CCA/MCA occlusion, all wounds were sutured closed and the rats were allowed to recover from anesthesia. Sham-operated rats were subjected to dissection of the temporalis muscle, craniotomy to expose the MCA, and positioning of the wire hook under the MCA. The hook was subsequently removed, and all wounds were sutured closed.

---

From the Cerebrovascular Disease Research Center, Department of Neurology (D.S., W.P.), Cornell University Medical College, New York, New York and the Department of Neurology (A.S.), Ohio State University, Columbus, Ohio.

Supported in part by National Institutes of Health grant 03346. A.S. was the recipient of National Institutes of Health training grant NS07141.

Address for reprints: Dr. A. Slivka, Ohio State University, 1654 Upham Drive, Room 431, Columbus, OH 43210.

Received March 27, 1989; accepted January 31, 1990.
Arterial blood pressure was monitored throughout the surgical procedure and then checked 2–4 hours after surgery, when the rats had recovered from anesthesia. PaO₂, PaCO₂, and arterial pH were measured just after tail artery cannulation, before MCA occlusion, and 2–4 hours after CCA/MCA occlusion. Glucose concentration and hematocrit were measured before MCA occlusion, and hematocrit was measured again before decapitation. During the surgical procedure, mean arterial blood pressure was maintained above 90 mm Hg by adjusting the halothane concentration delivered to the rat. PaO₂ was maintained above 80 mm Hg.

An intraperitoneal injection of L-carnitine (Chemical Dynamics Corp., South Plainfield, New Jersey) (16 mmol/kg, 1.2 M solution) was given 1 hour before MCA occlusion. This dose has been shown to double the concentration of carnitine in rat brains at 1 hour and nearly quadruple it by 24 hours. This dose is larger than that used experimentally for myocardial ischemia (0.5–1.2 mmol/kg). Control rats were injected with an equal volume (12.9 ml/kg) of normal saline. Rats were randomly allocated to treatment groups.

In our initial study (experiment 1), we compared carnitine- and saline-treated rats. Because of the high osmolarity of the carnitine solution, we were concerned that an observed reduction in infarct volume could be simply related to a reduction in brain edema. To control for this possibility, in experiment 2 we compared infarct volumes of rats treated with carnitine, saline, and mannitol; the latter was used as an osmotic control. Mannitol was injected intraperitoneally 1 hour before CCA/MCA occlusion in a dose equivalent to that of carnitine (16 mmol/kg, 1.2 M solution). Experiment 3 compared carnitine- and saline-treated rats because of the conflicting results of the first two experiments. Subsequent to experiment 3, we discovered that the method for removing the temporalis muscle using cautery may result in significant brain injury if care is not taken to avoid contact with bone. Since sham-operated rats were not included in experiments 1, 2, or 3, a fourth experiment was done with concurrent sham-operated rats to eliminate the possibility that the results were influenced by an uncontrolled factor associated with the surgical procedure.

At least 10 rats were included in each treatment group because this number has been found sufficient to avoid a type II error for a 25% reduction in infarct volume \((\beta=0.2, \alpha=0.05)\) in this model. Each experiment was completed over 2–3 weeks using rats delivered in a single shipment, from Charles River Laboratories, Inc. (Wilmington, Massachusetts) in experiments 1 and 2 and from Harlan Sprague Dawley Inc. (Indianapolis, Indiana) in experiments 3 and 4. The CCA and MCA were occluded in all four experiments by the same investigator. Experiments 1 and 2 were completed in the Cerebrovascular Disease Research Laboratory at Cornell University Medical College, and experiments 3 and 4 were done in a similarly equipped laboratory at the Ohio State University College of Medicine.

Whole-blood osmolality was measured using a vapor pressure osmometer (Wescor, Logan, Utah). In pilot studies, blood pressure and osmolality were monitored for up to 6 hours after the intraperitoneal injection of carnitine or mannitol. Neither drug affected blood pressure. Blood osmolality peaked 1–2 hours after injection and normalized by 4–6 hours. In experiment 2, blood osmolality was monitored before MCA occlusion, 2–4 hours after occlusion, and just before decapitation.

Rats were decapitated 24 hours after CCA/MCA occlusion. The brains were rapidly removed from the cranium and frozen in Freon over dry ice. Coronal sections 20 \(\mu\)m thick were cut at 500-\(\mu\)m intervals, fixed in 90% ethanol, and stained with hematoxylin and eosin. The area of infarction on each section was identified through image analysis (Quantimet 920, Cambridge Instruments, Inc., Buffalo, New York) for experiments 1, 2, and 3. For experiment 4, each section was magnified using a photographic lens and the infarcted area was traced onto paper. Each drawing was then retracted onto a digitizing tablet interfaced to an IBM personal computer (Video Image Analysis System, Ted Pella Inc., Redding, California), which computed infarct areas for each section. To calculate infarct volume, the infarct area of sequential sections was summed and then multiplied by the thickness between sections. Images were analyzed for each experiment by one investigator who was blinded to the treatment groups.

Mean±SD infarct volumes were computed for each treatment group. Results were analyzed using Student’s two-tailed \(t\) test for experiments 1, 3, and 4 and analysis of variance for experiment 2. Randomized-block-design analysis of variance was used to determine if a combined treatment effect was present for all four experiments.

**Results**

Physiologic data and the number of rats in each group are presented in Table 1. Before occlusion, anesthetized rats in all groups developed mild respiratory acidosis and blood pressure depression, which normalized by 2–4 hours after surgery. Hematocrit was unchanged over 24 hours in all treatment groups. For rats treated with carnitine and mannitol, serum osmolality was mildly elevated 2–4 hours after injection but returned to baseline levels by 24 hours.

Infarct volumes are shown in Table 2. In experiment 1, carnitine significantly \((p<0.05)\) reduced infarct volume compared with saline. However, replication of the study in three additional experiments revealed no effect of carnitine on infarct volume. Furthermore, combining the carnitine and saline groups from all four experiments yielded no statistical difference in infarct volume \(F_{10}=2.0, p=0.16\). Mannitol also had no apparent effect on infarct volume. Infarct volume for three sham-operated rats in experiment 4 was 3±4 mm³.
TABLE 1. Physiologic Variables for Rats With Focal Cerebral Ischemia

<table>
<thead>
<tr>
<th>Variable</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline (n=11)</td>
<td>Carnitine (n=12)</td>
<td>Saline (n=10)</td>
<td>Carnitine (n=10)</td>
</tr>
<tr>
<td><strong>Before occlusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>134±21</td>
<td>116±19</td>
<td>105±17</td>
<td>96±14</td>
</tr>
<tr>
<td>pH</td>
<td>7.28±0.05</td>
<td>7.27±0.05</td>
<td>7.26±0.03</td>
<td>7.24±0.03</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>100±12</td>
<td>105±17</td>
<td>116±20</td>
<td>105±14</td>
</tr>
<tr>
<td>Pco2 (mm Hg)</td>
<td>45±11</td>
<td>43±12</td>
<td>47±10</td>
<td>52±13</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>107±18</td>
<td>107±19</td>
<td>99±14</td>
<td>113±24</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>44±4</td>
<td>45±4</td>
<td>44±4</td>
<td>44±2</td>
</tr>
</tbody>
</table>
| Osmolality (mmol/kg) | ... | ... | 324±27 | 321±20 | 334±20 | ... | ... | ... | ...
| **After occlusion** |              |              |              |              |                |              |              |              |              |
| 2-4 hours |              |              |              |              |                |              |              |              |              |
| MABP (mm Hg)     | 180±12       | 178±27       | 162±30       | 148±15       | 157±7          | 177±19       | 154±17       | 203±14       | 176±14       |
| pH               | 7.35±0.04    | 7.31±0.05    | 7.37±0.06    | 7.28±0.03    | 7.28±0.05      | 7.32±0.03    | 7.27±0.04    | 7.35±0.05    | 7.30±0.05    |
| PaO2 (mm Hg)     | 92±13        | 96±9         | 96±12        | 97±17        | 107±24         | 99±18        | 115±10       | 98±14        | 104±18       |
| Pco2 (mm Hg)     | 37±6         | 34±11        | 38±5         | 44±8         | 40±7           | ...          | 37±5         | 38±4         | ...          |
| Osmolality (mmol/kg) | ... | ... | 328±20 | 343±19 | 345±37 | ... | ... | ... | ...
| 24 hours |              |              |              |              |                |              |              |              |              |
| Hematocrit       | 46±3         | 48±6         | 47±3         | 47±2         | 45±3           | 44±4         | 46±5         | 49±1         | 49±2         |
| Osmolality (mmol/kg) | ... | ... | 330±25 | 343±19 | 345±37 | ... | ... | ... | ...

MABP, mean arterial blood pressure. Data are mean±SD.

After CCA/MCA occlusion, mean arterial blood pressure and arterial pH were slightly lower in carnitine-treated rats than in saline-treated controls in experiments 2, 3, and 4. To determine if these differences influenced infarct volume and possibly masked a beneficial treatment effect, we calculated correlation coefficients for mean arterial blood pressure and arterial pH versus infarct volume for carnitine- and saline-treated rats from all four experiments. No correlation could be demonstrated between blood pressure and infarct volume (r=—0.01, p=0.4). This suggests that arterial pH and mean arterial blood pressure, within the ranges normally observed 2-4 hours after occlusion in this model, do not affect infarct volume.

Discussion

Changes in the concentrations of carmitine, long-chain acyl-CoA, and long-chain acylcarnitine in ischemic myocardium parallel those in ischemic brain. Carnitine treatment ameliorates these changes and improves function in ischemic hearts. Yet, we were unable to demonstrate that carmitine consistently influences infarct volume in our model of focal stroke.

If carmitine truly decreased infarct volume, as suggested in experiment 1, we should have been able to replicate the results in subsequent experiments. Therefore, we believe that the treatment effect noted in experiment 1 represents a type I error. The probability of detecting a false-positive result in two or more independent experiments can be estimated by multiplying the number of experiments (n) by α. When the experiments are independent, the exact probability is given by 1—(1—α)ⁿ. For example, with four experiments and an α of 0.05, the probability of committing a type I error is 19%. Naturally, the probability of the first experiment representing a type I error is only 5%. The absence of even a trend in favor of the treatment in experiments 2, 3, and 4 and the lack of a significant treatment effect after combining the carnitine-treated and control rats of all four experiments further support the conclusion that the results of experiment 1 occurred by chance.

Another possible (though much less tenable) explanation for the positive treatment effect noted in experiment 1 is that the beneficial effects of carmitine depend on the severity of the ischemic insult. This is a difficult premise to prove since there is no way to predict which group will have the larger mean infarct volume. However, despite the large control mean infarct volumes in experiments 2, 3, and 4, these volumes clearly do not represent complete infarction of the MCA territory. The largest infarct volumes achievable with this model through combined CCA/MCA occlusion and hypotension are in the range of 280-320 mm³. Furthermore, we have successfully
reduced infarct volume in this model through pharmacotherapy with nimodipine.\textsuperscript{15} In the latter study, the range of infarct volumes in control rats was similar to that seen in this series of experiments. For these reasons we do not believe that the positive results in experiment 1 can reasonably be attributed to the premise that carnitine is efficacious only in the setting of less severe ischemia.

Why carnitine appears to be protective in myocardial ischemia and not in focal cerebral ischemia may simply reflect the end points measured. We used histologically determined infarct volume as our outcome. The experimental and human studies reporting protective effects of carnitine in myocardial ischemia found improvement in myocardial function and a decrease in ischemia-induced ventricular fibrillation but did not assess infarct size histologically.\textsuperscript{8-11} The protective effects reported in myocardial function may thus represent an effect on surviving myocardium rather than an actual reduction in infarct size.

Unlike carnitine, mannitol has been used to treat focal cerebral ischemia. Yoshimoto et al\textsuperscript{16} reported a significantly decreased frequency of infarcts in dogs subjected to temporary brain ischemia and treated with 2 g/kg i.v. mannitol compared with untreated dogs. Mannitol given at 1.2 g/kg i.v. at the time of MCA occlusion in cats significantly attenuated ischemic neuronal damage for as long as 6 hours after occlusion.\textsuperscript{17-19} When 0.5 g/kg i.v. mannitol was given immediately before and after MCA occlusion in cats, ischemic injury was significantly reduced 12 hours after occlusion.\textsuperscript{20} Despite the apparent effectiveness of mannitol for up to 12 hours after MCA occlusion, these same studies demonstrated that mannitol did not alter pathologic changes seen 48 hours after MCA occlusion.\textsuperscript{19,20} Our study supports these latter findings that 2.9 g/kg i.p. mannitol has no significant effect on infarct volume 24 hours after the ischemic insult.

In addition to demonstrating that neither carnitine nor mannitol reduces infarct volume in this model, several caveats regarding the use of focal stroke models for testing drug efficacy were highlighted by this study. Our experience with focal ischemia in rats indicates that infarct volumes in a series of control animals may vary over time, especially in normotensive strains.\textsuperscript{12} This variation occurs despite a single investigator performing the surgery under the same laboratory conditions. Since we have no way to predict group variation in mean infarct volume, concurrent randomized controls should be used with each group of treated animals. Our results also suggest that confidence in a single experiment demonstrating a statistically significant pharmacologic effect should be tempered until the experiment has been replicated at least once.

Acknowledgments

We are grateful to Arthur Cooper for his helpful suggestions and to Steve Huber and Tim Costigan for their statistical assistance. We also thank Ms. Abigail Sarokin, Tibitha Harris, and Bobbie Swank for their help in typing the manuscript.

References

1. Schwartz A, Wood JM, Allen JC, Bornet EP, Entman ML, Goldstein MA, Sordahl LA, Suzuki M, Lewis ML: Biochemical and morphologic correlates of cardiac ischemia: I. Membrane systems. \textit{Am J Cardiol} 1973;32:46-61


8. Foits JD, Shug AL, Koke JR, Bittar N: Protection of the ischemic dog myocardium with carnitine. \textit{Am J Cardiol} 1978;43:1209-1214


11. Thomsen JH, Shug AL, Yap Vu, Patel AK, Karras TJ, DeFelice SL: Improved pacing tolerance of the ischemic human myocardium after administration of carnitine. \textit{Am J Cardiol} 1979;43:300-306


\textbf{Key Words} • carnitine • cerebrovascular disorders • rats
Carnitine treatment for stroke in rats.
A Slivka, D Silbersweig and W Pulsinelli

Stroke. 1990;21:808-811
doi: 10.1161/01.STR.21.5.808

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/21/5/808

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in
Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office.
Once the online version of the published article for which permission is being requested is located, click Request
Permissions in the middle column of the Web page under Services. Further information about this process is
available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/