Effects of Citicoline Combined With Thrombolytic Therapy in a Rat Embolic Stroke Model

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Background and Purpose—We sought to evaluate the effects of the combination of cytidine-5′-diphosphocholine (citicoline) and thrombolysis on infarct size, clinical outcome, and mortality in a rat embolic stroke model.

Methods—Eighty-three Sprague-Dawley rats were embolized in the carotid territory with a single fibrin embolus and randomly assigned to the following treatment groups: (1) control (saline), (2) citicoline 250 mg/kg, (3) citicoline 500 mg/kg, (4) recombinant tissue plasminogen activator (rtPA) 5 mg/kg, (5) rtPA 5 mg/kg plus citicoline 250 mg/kg, and (6) rtPA 5 mg/kg plus citicoline 500 mg/kg. rtPA was administered as a continuous intravenous infusion over 45 minutes starting 45 minutes after embolization; citicoline was given intraperitoneally 30 minutes and 24, 48, and 72 hours after embolization. At 96 hours, the brains were fixed and stained by hematoxylin-eosin, and infarct volumes were measured. Neurological scores were determined daily.

Results—The median infarct size, measured as percentage of the affected hemisphere, in the control group was 37% (interquartile range, 26% to 69%) compared with 22% (5% to 52%; P=NS) in group 2, 11% (5% to 34%; P=NS) in group 3, 24% (12% to 31%; P=NS) in group 4, 11% (3% to 22%; P=0.02) in the combined group 5, and 19% (9% to 51%; P=NS) in group 6. The infarct size was significantly reduced in the combined citicoline+rtPA–treated groups to a median of 13% (5% to 30%; P<0.01). Citicoline 500 mg/kg and citicoline combined with rtPA also promoted functional recovery.

Conclusions—These results demonstrate that the combination of low-dose citicoline and rtPA significantly reduced infarct size in this focal ischemia model. (Stroke. 1999;30:1464-1471.)

Key Words: cerebral ischemia, focal ■ neuroprotection ■ thrombolytic therapy ■ rats

Cytidine-5′-diphosphocholine (citicoline; CIT) administered exogenously provides both choline and cytidine, which serve as substrates for the synthesis of phosphatidylcholine, a primary neuronal membrane component. It is thought to promote membrane synthesis and repair, which is essential for recovery from stroke.1,2 CIT and its metabolites (choline and cytidine) contribute to the formation of acetylcholine, nucleic acids, and proteins.3 In models of transient hypoxia, CIT reduced production of free fatty acids, a tissue-toxic byproduct of membrane breakdown secondary to an ischemic insult.4 Additionally, CIT improved neurological outcome4 and increased survival time in global chronic hypoxia.5 In models of temporary focal ischemia, CIT reduced the size of cerebral infarcts6–7 and extended the duration of ischemia required to produce a given behavioral deficit or infarct size.8 A neuroprotective effect of CIT was reported in clinical studies of CIT-treated older (aged 50 to 85 years) subjects in which cognitive9–11 and behavioral parameters improved.12 In patients with stroke, CIT was associated with neurological improvements.1,13–16 Administration of CIT to stroke patients within 24 hours of symptom onset was associated with a more rapid recovery of neurological function and a greater proportion of patients with no residual neurological deficit in a recently completed clinical trial.17 In a subset of these patients, a reduction of the volume of the ischemic injury after stroke measured by diffusion-weighted MRI was demonstrated in 7 of 8 patients treated with CIT compared with 1 of 4 placebo-treated patients.18

Thrombolytic therapy has been studied extensively in experimental stroke and clinical trials19–23 and was approved in the United States for stroke treatment within 3 hours of symptom onset in selected patients in 1996.24 The results from the clinical trials were summarized in a meta-analysis; the Cochrane Systematic Reviews suggested a net long-term benefit for death and dependency with thrombolysis.25 However, the short time window and the increased risk of severe intracranial bleeding complications reported in a number of trials26–30 tend to limit the clinical use of thrombolytic therapy.

It therefore seems rational to explore the combination therapy of recombinant tissue plasminogen activator (rtPA),...
given at doses that are less likely to cause serious adverse events, and a neuroprotective agent that may widen the therapeutic window. The combination of thrombolytic agents with neuroprotective agents has been attempted, and some encouraging results have been reported in experimental stroke, although there is no convincing clinical evidence that any neuroprotective drug is effective in reducing size of infarction. To evaluate the effect of the combination of CIT and thrombolysis, CIT was administered in 2 different doses, alone and in combination with rtPA, and the effect on infarct size was compared in a rat embolic stroke model. The doses and time of administration of CIT and rtPA were chosen at suboptimal levels based on prior studies to evaluate a potential synergy between the drugs.

Materials and Methods

All experiments were approved and conducted according to the guidelines by the Animal Research Committee of Copenhagen University (No. 1996–101–103). Ninety male Sprague-Dawley rats (Møllegaard Breeding Center, Copenhagen) weighing 340 to 380 g were used. The rats were kept in a 12-hour light/12-hour dark cycle at a constant temperature of 22 ± 1°C and were fasted overnight with free access to water before the surgical procedures.

Anesthesia and Surgical Procedure

Anesthesia was induced with subcutaneous injection of fluanison (3 mg/kg) and fentanyl (1 mg/kg) (Hypnorm; fluanison 10 mg/mL and fentanyl 0.315 mg/mL; Janssen-Cilag) followed by a subcutaneous injection of 0.015 mg atropine and an intraperitoneal injection of 2.5 mg/kg diazepam (Apopenzam 5 mg/mL; Apothekernes Laboratorium). When necessary, anesthesia was prolonged with one third of the initial dose of fluanison and fentanyl. The right femoral artery was catheterized with a polyethylene tube (PP25; ID, 0.4 mm) for monitoring blood pressure and for sampling blood for analysis of arterial blood gases and glucose, and the right femoral vein was catheterized for intravenous drug administration. For the operation and for the first half hour after reversal of the anesthesia, body temperature was maintained at 37.0 ± 0.5°C with a heating lamp controlled by a thermostat connected to a temperature probe in the rectum.

The carotid operation was performed as described previously. Briefly, the right common carotid artery and the right external carotid artery (ECA) were exposed, and after ligation of extracranial branches of the internal carotid artery (ICA) and ECA, a polyethylene catheter (PP25) was inserted in the ECA. Clotting of the ECA was avoided by the continuous infusion (0.5 mL/h) of heparinized saline (5 IU/mL). After the second angiography, catheters were removed and vessels ligated. The femoral and neck wounds were closed, and the animals received isotonic saline 3.5 mL IP. Anesthesia was reversed with an injection of naloxone 0.3 mg/kg SC (Narcanti 0.4 mg/mL; Du Pont Pharma).

Embolization

Blood (250 μL) was aspirated from the femoral artery into a 1.0 mL 28-gauge disposable insulin syringe and injected through a 1.5-cm-long polyethylene catheter (PP10; ID, 0.28 mm; Polystan, Værløse) with another syringe containing 50 μL of human thrombin (Topostasin 2.5 IU/mL; Roche). A clot suspension was prepared by mixing the rat arterial blood with thrombin. The suspension was kept in constant motion by alternate movements from one syringe to the other for 3 minutes. By this procedure, small fibrin-rich arterial-like microthrombi were produced under high-pressure conditions. The syringes were left standing with closed compartments for 20 minutes. Then the suspension of microclots was injected into a 1.0-mm-long air-filled polyethylene catheter (PP10; ID, 0.28 mm). By this procedure, the microclots in the suspension aggregated into several visible clots of variable length. Clot material was transferred through a 60-mm piece of silicone (ID, 0.5 mm) to another 1.0-mm-long polyethylene catheter (PP10; ID, 0.28 mm) filled with saline. A piece of the catheter (100 mm) containing a single clot with a length of 4 mm (0.25 mm²) was selected for embolization. The obtained macro clot consisted of a fibrin mesh with a few corpuscular blood elements as determined by light and electron microscopy. The embolus was then injected through the ECA catheter, which was flushed gently for 30 seconds with 0.3 mL of isotonic saline. The common carotid artery was occluded by a temporary ligature, and the carotid bifurcation was inspected to avoid excessive dilatation of the ECA during the launching and flushing procedure.

Angiography

Carotid angiography was performed with the use of a high-resolution angiographic system (Philips SRO 03/100) with a small focus spot of 0.15 mm² and a large focus spot of 1.5 mm². Exposure data were 70 kV, 14 mA, and 0.4 seconds. One anteroposterior view with a focus object distance of 31.5 cm and a focus film distance of 141.5 cm, giving a linear magnification of 4.5, was done immediately and 2 hours after embolization. At each time point, a bolus of 0.20 mL heparinized (5 IU/mL) ioxolom (Omniaque, 300 mg/mL; Nycomed) was injected through the catheter. Angiograms were processed on high-resolution films (Retina XOE; Fotochemische Werke). The occlusions were classified according to the following categories: 0, patent arteries; 1, middle cerebral artery (MCA) branch occlusion; 2, MCA stem occlusion; and 3, ICA occlusion at the entrance of MCA distal to the posterior communicating artery. Only animals with occlusion types 2 and 3 were included in the study. In addition, a post hoc evaluation of the angiograms was conducted by raters blind to the treatment regimen, and animals not fulfilling the angiographic inclusion criteria were excluded from the final analysis.

Physiological Parameters

Blood samples were drawn from the femoral artery for analysis of pH, PaO2, PaCO2, O2 saturation (ABL2; Radiometer), and plasma glucose (B-glucose photometer; Hemocue AB) before embolization and 2 hours after embolization. Rectal temperature was continuously monitored, and mean arterial blood pressure was measured before embolization and after treatment.

Experimental Protocol

The rats were randomly assigned to 6 groups (n = 15 per group). Group 1 served as a control and was treated with saline as a continuous intravenous infusion (5 mL/kg) over 45 minutes starting 45 minutes after embolization and with saline administered intraperitoneally (1 mL/kg) 30 minutes and 24, 48, and 72 hours after embolization. Group 2 (CIT250) received saline as a continuous intravenous infusion (5 mL/kg) over 45 minutes starting 45 minutes after embolization and 250 mg/kg of CIT intraperitoneally (250 mg/kg) 30 minutes and 24, 48, and 72 hours after embolization.

Group 3 (CIT500) received saline as continuous intravenous infusion (5 mL/kg) over 45 minutes starting 45 minutes after embolization and 500 mg/kg of CIT intraperitoneally (500 mg/kg) 30 minutes and 24, 48, and 72 hours after embolization. Group 4 (rtPA) received 5 mg/kg of rtPA (Actilyse, Boehringer Ingelheim) as continuous intravenous infusion (1 mL/kg) over 45 minutes after embolization and saline intraperitoneally (1 mL/kg) 30 minutes and 24, 48, and 72 hours after embolization. Group 5 (CIT250 + rtPA) received CIT as in group 2 combined with rtPA as in group 4. Group 6 (CIT500 + rtPA) received CIT as in group 3 combined with rtPA as in group 4.

Clinical Evaluation

Neurological examination and weighing were performed after the animals recovered from anesthesia and over the next 4 days once daily at 24-hour intervals. Neurological status was evaluated according to Bederson et al, modified by adding grade 4 for death to a 5-point scale: 0 = no neurological motor deficit, 1 = flexion of the forelimb contralateral to the injured hemisphere, 2 = reduced resistance against push toward the paretic side, 3 = spontaneously circling
toward the paretic side, and 4 = death within 96 hours. Animals dying before 96 hours were autopsied to establish the cause of death.

Neuropathological Examination

After the neurological examination was completed on day 5, the animals were anesthetized with halothane and perfused transcardially with 4% phosphate-buffered formalin (pH 7.2). Brains were carefully removed, post-fixed in a 4% phosphate-buffered formalin solution, dehydrated, embedded in paraffin, and cut at a 3-μm section thickness. From each brain, ~15 coronal sections with a distance of 0.6 mm between slices were obtained and stained with hematoxylin-eosin. Brains from animals dying spontaneously 3-8 hours after embolization were removed and immersion-fixed in 4% buffered formalin solution. Brains from animals dying before 8 hours after embolization were considered as having a non-evaluable infarct size and were only evaluated by a freeze technique to verify pathological changes. The histology of the affected hemisphere and infarcts was examined blind to treatment with a Leica M3Z stereo microscope with 10 to 40 magnification, depending on the need for necessary details. The infarct was defined as an infarct regardless of whether it was pannecrotic or consisted of selective neuronal necrosis. Eosinophilic neurons in the periphery of pannecrotic areas were considered tissue damaged by ischemia and were included in the infarcted area. To distinguish the acidophilic neurons from other neurons and get an impression of the existence of light and dark neurons, a magnification of 40 was often supplied with a magnification of 100. Areas with dark neurons without eosinophilic neurons were not considered infarcted tissue. The borders of the infarcted areas were determined and delineated with a pen, and the delineated sections belonging to each brain were then scanned with a flat-bed scanner (HP ScanJet 4P) linked to a specially designed image analysis program (Sidney Data) in an IBM Pentium personal computer. The volumes of the hemisphere and infarcts were determined semiautomatically by the computer program detecting the circumference of the brain and the delineated borders in each section. The spatial resolution on the digitized images of the brain was set to 81 dots per square millimeter for area calculations. Adjustments and verification of the different areas were performed manually on enlarged digitized computer images. The volumes were then calculated as the respective areas multiplied by the distance between sections in percentage of the affected right hemisphere. The infarcts were measured without correcting the infarct volume for edema because the histological preparation technique dehydrated the tissue, reducing the influence of edema.

Statistical Analysis

Nonparametric analyses were used because a nonnormal distribution of data was observed in subsets of data. The Kruskal-Wallis test was applied to test for overall significant differences of ordinal data between groups. If significant differences were detected by the Kruskal-Wallis test, a Mann-Whitney test was performed to identify which group was different from controls. For comparisons of 2 groups, the Mann-Whitney test was performed for unpaired observations and the Wilcoxon signed rank test for paired observations. Binomial data (mortality rates, angiographic data) were compared by Fisher’s exact probability test. All probability values were corrected for multiple comparisons with the Bonferroni adjustment. The Spearman rank order correlation test was used for ranked paired observations. Corrected values of $P<0.05$ were considered significant. All calculations were performed on an IBM Aptiva personal computer with the use of a commercially available statistical software program (Sigma Stat version 2.03 for Windows).

Results

Ninety embolized animals were included in the study and randomly assigned to 1 of 6 treatment groups, as described in Materials and Methods. Of the 90 animals, 83 animals completed the study. Seven animals were excluded after embolization: 2 animals were excluded because of complications during surgery not related to treatment, 3 did not fulfill the angiographic inclusion criteria at a second blind angiographic review of the primary angiography, 1 animal was excluded because of missing second angiographic data, and 1 was excluded because of abnormal physiological variables.

All animals developed right hemisphere infarcts predominantly located in the right MCA territory. Infarction was less frequent in the hippocampus supplied by the posterior cerebral artery. Infarction was not found in the right cingulate cortex supplied by the anterior cerebral artery, except in 2 spontaneously dead animals with edema. In 1 animal, infarction was observed in the basal ganglia contralateral to the embolized hemisphere. No other contralateral infarction was observed. Petechial hemorrhagic transformation of the infarcts was observed in 7 animals, including 4 that died prematurely; 6 of the animals were in the rtPA-treated groups, and 1 was in the control group.

Clinical Outcome

Premature death occurred in 5 animals in the control group (36%), compared with 3 in the CIT250 group (20%), 2 in the CIT500 group (15%), 2 in the rtPA group (15%), 1 in the CIT250 + rtPA group (7%), and 3 in the CIT500 + rtPA group (21%). Except for 1 animal in the rtPA group that died within 6 hours after embolization, all animals died between 12 and 48 hours. There was no significant difference between the mortality rates in the different groups. The pooled groups treated with CIT alone (group 2+3) and the CIT + rtPA
combined groups showed a tendency toward a lower mortality.

No significant differences between the groups were observed in the neurological scores after the animals recovered from anesthesia ($P>0.9$, Kruskal-Wallis test), but an insignificant trend was found among treated animals compared with controls on day 5 ($P<0.08$, Kruskal-Wallis test; Figure 1).

All animals improved clinically during the study period. Within each group, including controls, the median clinical score on day 5 was significantly better than the median clinical score at day 1 ($P<0.01$, Wilcoxon test). The recovery was faster in the CIT500 and the CIT250+rtPA groups (Figure 1).

The body weight of animals in all groups declined from day 1 to day 3, then remained stable or improved (Figure 2). No significant differences in preoperative body weight were observed between the groups ($P>0.5$, Kruskal-Wallis test). On day 5 the Kruskal-Wallis test showed no significant differences of body weight between groups ($P>0.3$).

**Infarct Volume**

To obtain a complete evaluation of the effect of the different treatments, the results were analyzed both including and excluding the animals dying prematurely (Figure 3). The median values of the cortical, subcortical, and total infarct volumes as a percentage of the affected hemisphere and for animals surviving until euthanasia are shown in Table 1. There was no significant group difference of the median total infarct size among rats surviving 96 hours ($P>0.13$, Kruskal-Wallis test).

However, when the data were analyzed including the prematurely dead animals, significant group differences were found ($P<0.05$, Kruskal-Wallis test; Table 1 and Figure 3).

Compared with controls (37% [range, 26% to 69%]), a significant reduction of total infarct size was observed in the CIT250+rtPA group (11% [range, 3% to 22%]; $P=0.02$, Mann-Whitney test corrected by Bonferroni adjustment); no significant reduction in infarct volume was observed in the other treatment groups. When we pooled the data of all the CIT alone–treated animals (CIT250+CIT500), CIT given alone reduced the total infarct size from 37% (range, 26% to 69%) in controls to 19% (range, 5% to 50%; $n=28$; $P=0.054$, Mann-Whitney test adjusted by Bonferroni method), and CIT combined with rtPA reduced the infarct sizes even further to a median of 13% (range, 5% to 30%; $n=27$; $P=0.024$, Mann-Whitney test adjusted by Bonferroni method). One animal in the rtPA group died without having the brain fixed. The median infarct size in the spontaneously dead control animals was 71% (range, 55% to 72%; $n=5$; $P<0.01$, Mann-Whitney test) and significantly larger than the median infarct size in surviving controls ($P<0.01$, Mann-Whitney test). The median infarct size for all measured brains from spontaneously dead animals was 69% (range, 54% to 72%; $n=15$), which was significantly larger than for the surviving control animals (27% [range, 11% to 36%]; $n=9$; $P<0.001$, Mann-Whitney test).
Evaluation of Angiograms

The distribution of the occlusions immediately after embolization in the different groups immediately after embolization, but the difference in distribution was not statistically significant. Recanalization and redistribution of emboli distally in the cerebral arteries occurred spontaneously in both controls and treated animals during the first 2 hours of the experiment. This was most noticeable in the CIT500, rtPA, and CIT250+rtPA groups, especially in the CIT250+rtPA group, in which the frequency of complete recanalization compared with the control group increased from 0% (0/14) to 57% (8/14) (P<0.01, Fisher test) when compared with the pooled rtPA-treated groups, the frequency of complete recanalization compared with controls. When the 3 groups not receiving rtPA (including controls) were compared with the pooled rtPA-treated groups, the frequency of complete recanalization increased from 12% (5/42) to 37% (15/41) (P<0.01, Fisher test).

Correlations Between Clinical Outcome and Neuropathological Damage

The total infarct volume was highly correlated to the Bederson score on day 5 (all 96-hour animals; r=0.686, P<0.001) and to the net weight reduction on day 5 (all 96-hour animals; r=0.410, P<0.001). In other words, animals with smaller infarcts fared better functionally and regained weight more quickly.

Physiological Parameters

There were no systematic intergroup differences in the physiological parameters (all P>0.1, Kruskal-Wallis tests) except for the second measurement of pH (P<0.001, Kruskal-Wallis test). The CIT250+rtPA group was the only group with a significant (P<0.01, Mann-Whitney test adjusted by the Bonferroni method) lower second pH (median of 7.33 compared with a median of 7.40 in the control group). There was no significant difference between the physiological parameters of the animals that died prematurely and those that survived 96 hours (Table 3). When we compared the first set of parameters with the second, a fall in P O 2 and an elevation in P O 2 values in the CIT250+rtPA group was observed (P<0.05, Wilcoxon test). Similar changes were not observed in the other groups. There were no correlations between the physiological parameters and infarct size. No overt side effects of either rtPA or CIT were observed.

Discussion

The present study evaluated the effects of CIT given alone and combined with rtPA in an animal model of focal cerebral ischemia induced by embolization with autologous clots. The embolic model used in this study was created to imitate embolic human stroke, which varies in severity from transient ischemic attack to malignant MCA infarction. Even though the emboli used were of uniform size, the resulting arterial occlusions varied from small lesions to infarctions of the whole MCA territory with fatal edema formation. As in humans, occlusions of ICA could cause a range of infarcts from minimal to very large. This

**TABLE 1. Infarct Volume Expressed as Percentage of Affected Hemisphere**

<table>
<thead>
<tr>
<th>Group</th>
<th>Excluding Premature Dead Animals</th>
<th></th>
<th>Including Premature Dead Animals</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n Total</td>
<td>Cortical</td>
<td>Subcortical</td>
<td>n Total</td>
</tr>
<tr>
<td>Placebo</td>
<td>9</td>
<td>26.5 (11.0–35.6)</td>
<td>18.8 (6.2–24.1)</td>
<td>6.8 (4.5–11.7)</td>
</tr>
<tr>
<td>CIT250</td>
<td>12</td>
<td>18.8 (4.5–33.7)</td>
<td>15.9 (3.5–22.2)</td>
<td>4.2 (1.1–10.3)</td>
</tr>
<tr>
<td>CIT500</td>
<td>11</td>
<td>5.8 (1.2–19.9)</td>
<td>5.4 (0.7–15.0)</td>
<td>1.2 (0.6–4.8)</td>
</tr>
<tr>
<td>rtPA</td>
<td>11</td>
<td>23.7 (9.8–30.6)</td>
<td>12.6 (8.1–20.1)</td>
<td>6.6 (1.4–10.3)</td>
</tr>
<tr>
<td>CIT250+rtPA</td>
<td>13</td>
<td>9.5 (2.5–18.3)</td>
<td>7.9 (2.2–14.1)</td>
<td>2.5 (0.5–4.8)</td>
</tr>
<tr>
<td>CIT500+rtPA</td>
<td>11</td>
<td>11.0 (7.1–29.6)</td>
<td>6.2 (5.8–19.9)</td>
<td>5.1 (1.5–10.9)</td>
</tr>
</tbody>
</table>

Data are median values, with interquartile ranges in brackets.
P*<0.05, Mann-Whitney U test comparing treatment groups with control group and corrected for multible comparisons by Bonferroni adjustment.

**TABLE 2. Angiographic Findings**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immediately After Embolization</th>
<th>2 Hours After Embolization</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MCA (≥2)</td>
<td>ICA (≥3)</td>
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<tr>
<td>Placebo</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>CIT250</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>CIT500</td>
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<td>8</td>
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<tr>
<td>rtPA</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>CIT250+rtPA</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>CIT500+rtPA</td>
<td>14</td>
<td>4</td>
</tr>
</tbody>
</table>

Number and types of occlusion in each group are shown.
P*<0.001, Fisher’s exact test adjusted by Bonferroni method; comparison of complete recanalization with controls.
similarly to human stroke has the disadvantage of requiring large groups to obtain statistically significant effects.

The initial angiograms allowed selection of animals with occlusion of the MCA stem and the ICA, but the distribution of these 2 types of occlusion was unevenly balanced, with more initial MCA occlusion in groups 2 to 5. One could speculate that this may have influenced the finding of more initial MCA occlusion in groups 2 to 5. One could speculate that this may have influenced the finding of significant treatment effect. However, if the data analysis was restricted to the animals with ICA occlusions on the primary angiogram, the median total infarct volume was 41% in controls (n=11) and 22% in the combination group with CIT500 and rtPA (n=6; P=0.08, Mann-Whitney test). A similar trend was seen if the data analysis was restricted to the animals with MCA occlusion on the primary angiogram, where the median initial infarct volume was 27% in controls (n=3) and 5% in the CIT250+rtPA group (n=8; P=0.10, Mann-Whitney test). Thus, the significant reduction of median infarct volume in the combination therapy group was not due to a favorable outcome in a subset of animals but was present regardless of the type of occlusion on the first angiogram.

The animals that died prematurely were evaluated to ensure that no treatment would cause an excess fatality rate and result in false-positive treatment effect by allowing only subjects with small infarcts to survive. Characteristically, the brains from the animals that died prematurely had edema with compression of the ventricles, and premature death was probably caused by large infarcts with brain swelling. Small hemorrhagic transformations, observed primarily in the rtPA-treated animals, did not seem to contribute to fatal outcomes.

To more easily evaluate potential synergy between the 2 drugs, the doses for each drug were selected to have a suboptimal effect on infarct volume given alone. At the same time, a parallel series of experiments was conducted to evaluate the effect of a fully effective dose of CIT on infarct size, given alone and in combination with rtPA. In previous studies using a temporary focal occlusion model in rats, CIT significantly reduced infarct size at a dose of 500 mg/kg IP but not at a dose of 250 mg/kg IP.7 The findings with these doses of CIT in the present study are consistent with those in the temporary focal occlusion model. In the rat, rtPA needs to be given at doses 10 times higher than in humans to have equal effect.42,43 In our previous studies with rtPA, doses of 10 to 20 mg/kg were used.19–21,33,34,38 The 5-mg/kg dose used in this study was considered suboptimal in terms of recanalization capability, but with reduced risk of bleeding.

When a suboptimal treatment regimen of CIT (250 mg/kg) and rtPA were combined, the result was a significant reduction in infarct volume and a tendency for improvement in clinical outcome. This interaction between the agents appears to be additive. It is unclear why the combination of a more effective dose of CIT (500 mg/kg) with the suboptimal rtPA treatment regimen did not produce a similar or greater infarct size reduction than the lower dose combination. One explanation may be the large variation in infarct size within this group (9% to 51%) compared with other treatment groups (eg, 3% to 22% for the CIT250+rtPA group). Acknowledging the wide range of infarct sizes with this model and the need for large groups of animals, we also compared the effects of the combined CIT-alone groups with the combined CIT+rtPA groups. The combined groups treated with CIT alone showed a tendency to reduce infarct size, but infarct size was significantly reduced in the combined CIT+rtPA-treated groups when the dead animals were included in the analysis.

In conclusion, this study provides a basis for further investigation of the effects of CIT combined with rtPA for the treatment of ischemic stroke. Although histological assessment of neuropathological changes is ultimately the most predictive measure of potential therapeutic value of a drug,31

### TABLE 3. Physiological Parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Temperature, °C</th>
<th>MABP, mm Hg</th>
<th>PacO2, mm Hg</th>
<th>PaO2, mm Hg</th>
<th>Glucose, mmol/L</th>
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</thead>
<tbody>
<tr>
<td>Before embolization</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>37.0</td>
<td>73</td>
<td>7.42</td>
<td>40.5</td>
<td>96.3</td>
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<td>37.2</td>
<td>70</td>
<td>7.39</td>
<td>41.4</td>
<td>88.4</td>
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<tr>
<td>CIT500</td>
<td>37.0</td>
<td>71</td>
<td>7.38</td>
<td>41.0</td>
<td>91.4</td>
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<tr>
<td>rtPA</td>
<td>37.1</td>
<td>76</td>
<td>7.40</td>
<td>43.0</td>
<td>92.8</td>
</tr>
<tr>
<td>CIT250+rtPA</td>
<td>37.2</td>
<td>76</td>
<td>7.37</td>
<td>42.8</td>
<td>96.2</td>
</tr>
<tr>
<td>CIT500+rtPA</td>
<td>37.0</td>
<td>75</td>
<td>7.40</td>
<td>40.0</td>
<td>91.7</td>
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<tr>
<td>Before second angiography</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>37.3</td>
<td>82</td>
<td>7.40</td>
<td>38.6</td>
<td>86.5</td>
</tr>
<tr>
<td>CIT250</td>
<td>37.4</td>
<td>89</td>
<td>7.40</td>
<td>40.0</td>
<td>83.1</td>
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<td>CIT500</td>
<td>37.2</td>
<td>86</td>
<td>7.39</td>
<td>40.1</td>
<td>85.8</td>
</tr>
<tr>
<td>rtPA</td>
<td>37.0</td>
<td>83</td>
<td>7.40</td>
<td>40.0</td>
<td>86.3</td>
</tr>
<tr>
<td>CIT250+rtPA</td>
<td>37.3</td>
<td>80</td>
<td>7.33</td>
<td>42.3</td>
<td>92.4</td>
</tr>
<tr>
<td>CIT500+rtPA</td>
<td>37.4</td>
<td>85</td>
<td>7.36</td>
<td>38.2</td>
<td>94.4</td>
</tr>
</tbody>
</table>

Data are median values. MABP indicates mean arterial blood pressure.
The CIT250+rtPA was the only group with a significant (P<0.01, Mann-Whitney test corrected by Bonferroni adjustment) lower second pH (median 7.33 compared with 7.40 in the control group).

No other significant differences were observed between the groups (all P>0.1, Kruskal-Wallis tests).
the demonstration that CTP+rtPA also promotes functional recovery supports the assumption of a potential benefit of this combined therapy.

Acknowledgments
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References
The accompanying article reports experimental results of combined neuroprotective agent and thrombolytic therapy in the embolic stroke model. The study design seems to be carefully considered, so that one can examine the effect of various combination doses of rtPA and citicoline (CIT). In addition, appropriate statistical analyses were performed, including adjustments for multiple tests followed by an overall test for each variable.

The median infarct volumes reported by the authors can be conveniently displayed in the following $2 \times 3$ table. The Table allows the readers to see an apparent effect of CIT dose alone, rtPA dose alone, and combination rtPA and CIT dose on median infarct volumes.

<table>
<thead>
<tr>
<th>CIT, mg/kg</th>
<th>0</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>rtPA, mg/kg</td>
<td>0</td>
<td>37.4</td>
<td>22.3</td>
</tr>
<tr>
<td>5</td>
<td>24.3</td>
<td>10.7</td>
<td>18.8</td>
</tr>
</tbody>
</table>

Although the sample size in each group is not large, the results indicate linear effects of rtPA alone, CIT alone, and CIT and rtPA combination, except for the CIT 500 mg/kg and rtPA combination. First, although only the rtPA+CIT250 group is significantly different from the control, it is certain that the difference between the CIT500 group and the rtPA+CIT250 group is not significant. Second, the result seems to suggest an interaction effect of CIT and rtPA. Similar results are observed for body weight change and Bederson scores. We hope that authors will perform further studies to confirm the superiority of the combination of the 2 agents and the possible interaction effect. Incidentally, it is interesting to note that spontaneously dead animals had large infarct volumes in each group. Consequently, the nonparametric analysis used by the authors would not have changed the results even if dead animals survived 4 days.

A concern the readers might have is the large variance in infarct volumes. Sometimes, part of a large variance may be due to the variances of uncontrolled factors in the model, such as age and weight of animals. Another reason is the large positive skew in the data. Note that the shape of distribution of the infarct volume for the 6 groups is about the same with a large positive skew. A positively skewed distribution is to be expected in infarct volumes.

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Effects of Citicoline Combined With Thrombolytic Therapy in a Rat Embolic Stroke Model
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