Graded Bioassay of Demonstration of Brain Rescue From Experimental Acute Ischemia in Rats

J. Aronowski, PhD; P. Ostrow, MD; E. Samways, BS; R. Strong, BS; J.A. Zivin, MD; J.C. Grotta, MD

Background and Purpose This study explored the correlation between duration of focal ischemia and infarct volume in spontaneously hypertensive rats as a measure of outcome after neuroprotective intervention.

Methods We used 2,3,5-triphenyltetrazolium chloride staining to discriminate infarcted tissue and calculate infarct volume 24 hours after temporary tandem common carotid/middle cerebral artery occlusion lasting 5 to 150 minutes. We used a graded bioassay described by logistic function and executed by computer program (ALLFIT) to evaluate changes in infarct volume after increasing durations of ischemia. The method allowed us to calculate the maximal infarct volume (Volmax) and the duration of ischemia before reperfusion producing half-maximal infarct size (T50). Hypothermia and the N-methyl-D-aspartate antagonist CNS-1102 begun after the onset of ischemia were tested for their ability to reduce Volmax and prolong T50 as analyzed by ALLFIT.

Results Volmax was 180.6±22.4 mm³ and T50 was 45.9±5.8 minutes in control rats. Hypothermia (30°C) applied during ischemia reduced Volmax by 66 mm³ and extended T50 by 50% (P<.05 for each comparison). CNS-1102, like hypothermia, extended T50 by 44% but did not have an effect on Volmax.

Conclusions Analysis of the changes of infarct size after increasing durations of ischemia indicates that although both were protective, the two treatments tested may exhibit different profiles of efficacy. This method of analyzing ischemia-induced damage may be very sensitive for studying the efficacy and possible clinical use of neuronal protective therapies for hyperacute stroke. (Stroke. 1994;25:2235-2240.)

Key Words • cerebral infarction • cerebral ischemia, focal • neuroprotection • reperfusion • rats

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Several factors can influence the results of preclinical trials of pharmacoprotection in animal models. The first factor is the time interval between onset of stroke and therapeutic intervention. The shorter the interval between onset of ischemia and start of therapy, the better the protection that can be expected. Selection of the dose and route of drug administration, allowing for optimal drug plasma levels, also directly influences outcome, which may be particularly important during the testing of new drugs without available data characterizing their pharmacokinetic and pharmacodynamic properties. Finally, ischemia that lasts several hours may cause such extensive damage that drugs will offer no protection. This last factor is very often overlooked in the design of studies that test the efficacy of new anti-ischemic pharmacotherapies, which often leads to an incorrect conclusion regarding therapeutic efficacy (or the lack thereof). To understand the importance of the duration of ischemia as a factor that contributes to outcome from preclinical animal studies evaluating the efficacy of new drugs, we designed an experiment in which we subjected hypothermic and N-methyl-D-aspartate (NMDA) receptor antagonist (CNS-1102)–treated rats to variable durations of ischemia, after which we measured and compared the infarct sizes using a graded bioassay method.

Materials and Methods

Production of Ischemia

Focal ischemia in male spontaneously hypertensive rats (weight, 252 to 294 g) was induced by tandem left MCA and left common carotid artery (CCA) occlusion. The animals
were fasted overnight with free access to water and then anesthetized with an injection of chloral hydrate (0.5 g/kg IP). The femoral artery was cannulated with a polyethylene catheter for blood pressure measurement. Core body temperature was recorded with a microprobe thermometer (Yellow Springs Instrumental) inserted 6 cm deep into the rectum. Brain temperature in randomly selected animals in both experimental and control groups was recorded from corup striation contralateral to the side of ischemia, as previously described. Additionally, temperature was monitored from the right temporalis muscle by means of a digital microprobe (Omega Engineering model 410T-B with 0.1°C resolution). The temporalis muscle temperature during ischemia and the first hour of reperfusion in the control and CNS-1102 groups at any time of the experiment was maintained between 36.2°C and 36.8°C with a heating lamp and warming blanket. Hypothermia (30°C) was induced by placing the rats on crushed ice 5 minutes before surgery. The left CCA was isolated through a midline incision and tagged with a suture. An incision was made through the temporalis muscle perpendicular to a line drawn between the external auditory canal and the lateral canthus of the left eye. Under direct visualization with the surgical microscope, two burr holes were made with a hand-held drill: a 1 x 3-mm rectangular burr hole, situated 2 to 3 mm rostral to the fusion of the zygomatic arch with the squamosal bone to expose the left MCA rostral to the rhinal fissure, and a 1-mm round burr hole for CBF measurement 4 mm dorsal to the MCA exposure. The underlying bone was irrigated constantly with normal saline. A laser Doppler flow probe (Vasomedic) was placed over the saline-irrigated intact dura to measure baseline CBF. The beveled edge of a 23-gauge hypodermic needle was used to pierce and open the dura along the entire length of the rectangular burr hole. The needle from a 10-0 BV 130-3 Ethicon neurosurgical suture was passed out under the MCA in a position proximal to the major cortical branching of the MCA but distal to the lenticulostriate arteries. To induce ischemia the needle was rotated clockwise so that one end pointed toward the vertex forming a right angle to the burr hole, thus lifting the MCA above the cortical surface and interrupting flow. Immediately after MCA occlusion, the left CCA was occluded with an atrumatic Helezon aneurysm clip. Interruption of flow through the MCA was inspected under the microscope and verified by CBF measurement from the laser Doppler flowmeter. At the end of the desired period of ischemia and during the first few minutes of reperfusion, the arterial blood pressure (lowered by chloral hydrate) was tracheotomized and the preischemic control CBF was measured. After a 5-minute infusion of 0.1% phenylephrine (10 to 20 μg/kg per minute). CBF changes associated with this treatment were continuously monitored.

Experimental Groups

Animals were divided into five experimental groups. The first two groups were formed from animals that were subjected to a fixed 120 minutes of ischemia. Rats in these two groups were analyzed in a separate study several months before the introduction of the graded bioassay method of analysis. Group 1 (n=10) was treated with CNS-1102, and group 2 (n=11) was its saline-treated control. CNS-1102 was administered first as a bolus of 0.5 mg/kg IV, immediately followed by intravenous infusion (with an osmotic pump [Alza Corporation]) at a constant rate of 0.345 mg/kg per hour, with a flow rate of 0.01 mL/min. The treatment was initiated 30 minutes after the induction of ischemia and continued for the next 24 hours. The remaining three groups, which were formed subsequently to negative results of the experiments with groups 1 and 2, consisted of control, hypothermia-, and CNS-1102-treated rats; they were subjected to varying durations of ischemia ranging from 5 to 150 minutes. Group 3 contained 23 control (untreated) rats. Group 4 contained 20 rats subjected to hypothermia. The brain temperature of rats in this group was lowered to 29.9±0.2°C (SEM) 5 minutes before tandem MCA/CCA occlusion and was maintained at this temperature for the entire duration of ischemia. On reperfusion the temperature was allowed to return spontaneously to physiological values (approximately 25 to 30 minutes). Group 5 contained 14 rats treated with CNS-1102. Treatment in this group was initiated 15 minutes after the induction of ischemia, with the dosages as in group 1. Unlike group 1, CNS-1102 in this group was delivered from a syringe pump (Sage Instruments) instead of an osmotic pump and for 3 hours instead of 24 hours. Animals from each experimental group were killed 24 hours after ischemia for measurement of infarct volume.

Infarct Volume Estimation

All measurements of infarct volume in all groups were performed 24 hours after the induction of ischemia. Rats were killed under chloral hydrate anesthesia by intracardiac perfusion with 100 mL of 0.9% saline delivered under a constant pressure of 130 mm Hg. The brains of the animals in groups 1 and 2 were additionally fixed by intracardiac perfusion with 50 mL of phosphate-buffered 10% formalin to prepare them for histological analysis. Formalin-fixed brains were dehydrated, cleared, and embedded in paraffin. Serial sections were cut at 5 μm in the coronal plane, and every 100th section (0.5-mm intervals) was stained with a combination of hematoxylin-eosin and Luxol fast blue to demarcate infarcted tissue. The unfixed brains (groups 3, 4, and 5) were cooled immediately after saline perfusion in phosphate-buffered saline and sectioned into 2-mm coronal sections with a Jacobowitz brain slicer. The infarcted regions of each of the sections were visualized by 30-minute staining at room temperature in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate-buffered saline. TTC is an indicator of mitochondrial respiratory enzymes and is considered to be a rapid and convenient stain for detection of infarcted tissue. Recent work has described an excellent correlation between TTC staining and conventional time-consuming histological examination with hematoxylin-eosin staining. TTC-stained sections were transferred to phosphate-buffered 10% formalin before the infarct volume measurement.

Morphometric determination of the outlined infarcted area of each section was performed with a computer-based Drexel University (DUMAS) image analyzer (groups 3, 4, and 5), or DCTV (Digital Creations) frame grabber (groups 1 and 2), calibrated to express measurement in square millimeters. The direct total infarct volume (expressed in cubic millimeters) was calculated by summing the infarct area of sequential sections and multiplying by the interval thickness between sections.

Correlation Between Time of Ischemia and Infarct Volume

The first analysis (group 1 versus group 2) was performed to answer a simple question: Is there any statistical difference between the infarct volume produced by 2 hours of ischemia in animals treated with CNS-1102 versus placebo? The difference between these two groups was assessed with Student's t test. The second analysis used a general computerized method, developed by De Lean and coworkers and recently described by Zivan and Waud, to describe the correlation between the duration of ischemia and the infarct size in terms of basal and maximal responses, ED50, and curve shape and steepness. The computer program (ALLFIT) used to perform this analysis uses the logistic function $y = \frac{(a-d)}{1 + (e + \frac{x}{b})} + d$, where $y$ is the infarct volume, $x$ is the duration of ischemia, $a$ is the response when $x=0$, $d$ is the maximal infarct volume (hereafter labeled $V_{inf}$), $b$ is a slope "factor" that determines the steepness of the curve, and $c$ is the ED50 (the duration of ischemia resulting in half-maximal infarct volume, hereafter labeled $T_{0.5}$). This program was developed for the simultaneous fitting of families of sigmoidal dose-response curves and was obtained from the Laboratory of Theoretical and Physical Biology at the National Institutes of Health. The statistical difference in $T_{0.5}$ and
Physiological Variables in Untreated, Hypothermic, and CNS-1102-Treated Rats Before and After Anesthesia, During Common Carotid/Middle Cerebral Artery Occlusion, and 10 Minutes After Reperfusion

<table>
<thead>
<tr>
<th>Physiological Parameters</th>
<th>Untreated</th>
<th>Hypothermic</th>
<th>CNS-1102</th>
</tr>
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<tbody>
<tr>
<td>Preischemia</td>
<td>Baseline</td>
<td>Ischemia</td>
<td>Reperfusion</td>
</tr>
<tr>
<td>Core CBF</td>
<td>123±9 (6)</td>
<td>4±1 (7)</td>
<td>60±7 (6)</td>
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<tr>
<td>Hypothermia</td>
<td>117±20 (5)</td>
<td>4±1 (5)</td>
<td>49±13 (4)</td>
</tr>
<tr>
<td>CNS-1102</td>
<td>111±16 (7)</td>
<td>4±1 (12)</td>
<td>66±22 (8)</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td>131±9 (19)</td>
<td>116±13 (16)</td>
<td>130±14 (15)</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>132±11 (19)</td>
<td>92±16 (8)</td>
<td>107±22 (15)</td>
</tr>
<tr>
<td>CNS-1102</td>
<td>130±9 (12)</td>
<td>118±10 (12)</td>
<td>130±14 (11)</td>
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CBF indicates cerebral blood flow; MABP, mean arterial blood pressure. Relative values of CBF were obtained from the numeric display on the laser Doppler flowmeter. Values are mean±SEM of (n) analyzed animals.

Results

Physiological Parameters

There were no significant differences between ischemic control, hypothermic, and CNS-1102-treated rats with respect to physiological variables including weight, Pco₂, and Po₂. Mean arterial blood pressure, CBF, and temporalis muscle temperature during ischemia and the first hour of reperfusion were not significantly different between control and CNS-1102-treated rats. In hypothermic animals, mean arterial blood pressure was significantly lower than that in control and CNS-1102-treated animals (Table). In addition, pH values that were indistinguishable between control and CNS-1102-treated rats were slightly higher in hypothermic animals compared with control (7.36±0.03 versus 7.30±0.05).

For the temperature analysis, we established a correlation between brain (measured from corpus striatum) and temporalis muscle temperature. There was no significant difference between brain and temporalis muscle temperature obtained from 11 animals. The temperature in the temporalis muscle in control animals was 36.35±0.1°C, whereas for brain tissue it was 36.40±0.1°C. The temperature recorded from the temporalis muscle and the brain was also not significantly different in animals subjected to hypothermia (30.3±0.3°C for the temporalis muscle and 29.9±0.2°C for the brain). Temporalis muscle temperature in control and CNS-1102-treated rats was maintained between 36.2°C and 36.7°C during ischemia and the first 2 hours of reperfusion. Temporalis muscle temperature during ischemia in the hypothermic group was maintained between 31.2°C and 29.4°C. With the laser Doppler blood perfusion monitor, with the probe placed distal to the MCA occlusion, CBF was monitored for each rat to verify the cessation of blood flow and to confirm reperfusion after reversing the CCA/MCA occlusion (Table). Relative values of CBF obtained from the numeric display on the laser Doppler flowmeter are presented. Baseline CBF and mean arterial blood pressure values were obtained from rats under ether and before chloral hydrate anesthesia.

Chloral hydrate anesthesia alone significantly decreased CBF by 36±13% compared with baseline and also caused a significant decrease in mean arterial blood pressure by 23±7% in all animals. This depressing effect of chloral hydrate on mean arterial blood pressure was reversed during reperfusion by the continuous infusion of 0.1% phenylephrine. This protocol facilitated reperfusion and allowed for the standardization of reperfusion parameters. In preliminary studies we observed significant variabilities from rat to rat in reperfusion CBF values, which could potentially cause inconsistency of infarct volume. Relative CBF recorded from the ischemic cortex during CCA/MCA occlusion was indistinguishable among control, CNS-1102-treated, and hypothermic animals and was approximately 4.0, as indicated in the Table.

Determination of Infarct Volume Produced by 2 Hours of Ischemia

We measured infarct volume for analysis of group 1 (CNS-1102) and group 2 (placebo control) using hematoxylin-eosin and Luxol fast blue staining of a series of coronal brain sections, as described in "Materials and Methods." Treatment with CNS-1102 did not affect the size of infarct in these animals. Infarct volume produced by 2 hours of ischemia was 160.1±11.1 mm³ and 157.9±25.5 mm³ for CNS-1102- and placebo-treated animals, respectively (Fig 1).

Determination of Infarct Volume Produced by Variable Durations of Ischemia

The correlation between duration of ischemia and infarct volume was derived by means of the computer logistic function, as described in "Materials and Methods." Increased duration of ischemia resulted in a graded increase in infarct volume (Fig 2A). Five minutes of ischemia did not produce any changes detected by staining of the infarct zone. A noticeable infarct of 5 mm³, with the core localized approximately 2 mm posterior to the bregma, was first observed after 10 minutes of ischemia. The most dramatic increase in brain damage occurred between 20 and 50 minutes of ischemia, with a maximum achieved after approximately 60 minutes of ischemia.
CCA/MCA occlusion. The $T_{50}$ was 45.9±5.8 minutes, and predicted $V_{\text{max}}$ was 180.6±22.4 mm$^3$.

Effects of Hypothermia and CNS-1102 on Infarct Volume Produced by Variable Durations of Ischemia

A transient 7°C decrease in brain temperature induced 5 minutes before CCA/MCA occlusion significantly reduced brain damage. First, hypothermia dramatically extended to 20 minutes the duration of ischemia that the brain could withstand without having any measurable infarct (Fig 2B). In addition, the $T_{50}$ was significantly extended from 45.9±5.8 minutes in normothermic rats to 69.3±7.3 minutes in hypothermic rats ($P<.05$). This 50% increase in $T_{50}$ was accompanied by a 37% decrease in $V_{\text{max}}$ in hypothermic rats. The computed $V_{\text{max}}$ after hypothermia was 114.2±6.2 mm$^3$, 66 mm$^3$ smaller than the $V_{\text{max}}$ produced under normothermic conditions ($P<.05$).

Posttreatment with the noncompetitive NMDA-antagonist CNS-1102 also had a beneficial effect on the ischemia-induced volume of the infarct (Fig 2C). Similar to hypothermia, CNS-1102 significantly extended the duration of ischemia that would not produce any measurable infarct (Fig 2C). The infarct volume versus duration of ischemia correlation curve for CNS-1102 was significantly shifted to the right, with the $T_{50}$ extended by 20 minutes to 66.1±1.9 minutes compared with control animals ($P<.05$), indicating the efficiency of the drug in delaying damage produced by ischemia.

In contrast to hypothermia, CNS-1102 did not reduce $V_{\text{max}}$. Similar to results obtained after a fixed 2-hour duration of ischemia (groups 1 and 2) with Luxol fast blue staining (Fig 1), $V_{\text{max}}$ measured by TTC in CNS-1102-treated animals was 165.2±9.8 mm$^3$ and also was not different from $V_{\text{max}}$ in controls (Fig 2A and 2C). It appears from our data that CNS-1102 exerts its protective effect for only up to 70 minutes of ischemia. Any insult exceeding 70 minutes of CCA/MCA occlusion resulted in damage that was indistinguishable between control and CNS-1102–treated animals. Direct comparison between the experimental groups is demonstrated in Fig 3.

Discussion

Our study indicates that a graded bioassay correlating the duration of ischemia and infarct size is a very
efficient and sensitive method to study the efficacy of stroke cascade. Using this method we showed that both the noncompetitive NMDA antagonist CNS-1102 and 30°C hypothermia were effective in decreasing the severity of damage produced by reversible CCA/MCA occlusion.

It can be deceptively difficult to design an experiment to study the therapeutic usefulness of an anti-ischemic drug for clinical studies. Several factors, such as optimal dose, time of treatment (before versus after ischemia), and the severity of insult, may determine the outcome. Whereas dosing and the time of initiation of treatment represent well-recognized factors in the design of drug testing, severity of ischemia is not always taken into consideration. For example, in our previous study of the temporal analysis of changes in calmodulin immunostaining that correlate closely with subsequent histological damage, we found that reproducible cortical infarction appeared after occlusion of at least 1 hour and that maximal infarct developed after at least 3 hours of ischemia. Therefore, 2 hours of ischemia appeared to represent a justified, submaximal severity of insult to be used in the testing of a neuroprotective drug. However, when we tried to assess the neuroprotective properties of CNS-1102 on rats subjected to 2 hours of ischemia (Fig 1) before introduction of graded bioassay, we could not detect any histological protection. Since the dose of CNS-1102 used in our studies had already been shown to be effective in the suture model of MCA occlusion, we concluded that the negative result was most likely due to an excessive severity of the insult produced in our model. To verify this hypothesis, we used an analysis of infarct size produced by various durations of ischemia. Using this method, we decided to compare the efficacy of hypothermia and CNS-1102.

Hypothermia is recognized as one of the most effective therapies used to protect the brain from damage produced by ischemia. The mechanism by which hypothermia produces its beneficial effect is not fully understood, but several hypotheses, including changes in neurotransmitter release, protein synthesis, protein kinase C, or calcium/calmodulin-dependent protein kinase II activity, have been suggested. Glutamate receptor antagonists, especially antagonists of its NMDA subtype, are currently considered to be some of the most promising therapies for human stroke. Block of this receptor in vitro and in vivo is known to decrease excessive calcium influx into neurons overexposed by elevated extracellular glutamate concentration. Persistent excess intracellular Ca++ is perceived to initiate various biochemical cellular processes that lead to cell death. One of the new chemical groups of NMDA antagonists recently described is a derivative of the diarylguanidines. These compounds block NMDA receptor-coupled cation channels and block excitotoxicity induced by glutamate in vitro neuroprotection models. CNS-1102 (N-(1-naphthyl)-N'-(3-ethylphenyl)-N'-methyl guanidine) is a highly potent and selective noncompetitive NMDA antagonist with a relatively short plasma half-life as assayed in rats. It was shown recently that CNS-1102 is effective in reduction of infarct size after suture MCA occlusion in Sprague-Dawley rats. Since NMDA receptor antagonists are known to decrease body and brain temperature, in our preliminary study we first verified that the protection obtained after CNS-1102 treatment was not due to lowering of brain temperature. Similarly, changes in CBF are well known to directly determine infarct size. Measurements obtained with the laser Doppler flowmeter did not allow for simultaneous measurements of CBF in the penumbra and core of MCA arborization. However, analysis of the central core of the infarct did not detect any significant differences in CBF between control, hypothermic, or CNS-1102–treated animals, suggesting that changes in infarct volume were not due to any major flow improvements in the core of the infarct.

Although both treatments extended the T50, reduction of Volm, after hypothermia versus CNS-1102 treatment was significantly different. Both treatments were equally capable of reducing infarct size in rats subjected to a duration of ischemia no longer than 70 minutes. After the length of ischemia was increased above this duration, CNS-1102, unlike hypothermia, no longer exerted any neuronal protection compared with the control (untreated) animals.

Hypothermia not only extended the time that neurons could withstand ischemia before maximal damage occurred but also decreased maximal damage. The maximal damage produced in hypothermic animals reached a plateau similar to the control and CNS-1102 groups after approximately 1.5 hours and was 37% smaller than in CNS-1102–treated and control animals. It is possible that CNS-1102 treatment, in contrast to pretreatment with hypothermia, had less chance for more extended brain protection because it was initiated 15 minutes after induction of ischemia. It will be interesting to determine whether an NMDA receptor antagonist combined with hypothermia will further reduce infarct size. Moreover, extension of hypothermia into the first few hours of reperfusion could be helpful in answering the questions of how much damage caused by reperfusion exists, if any, and whether it can be reduced by this powerful treatment. Recent reports have been contradictory, however, regarding whether hypothermia during reperfusion can reduce ischemic damage.

In conclusion, our study demonstrates that a graded analysis of infarct size produced by variable durations of ischemia is a very powerful technique to test the overall profile of treatment efficacy. This method of analysis has the ability to discriminate between and compare treatments of different potencies for insults of graded intensities. If it is hypothetically assumed that certain drugs can be more effective after brief ischemia (helping to shift the T50 to the right more than other treatments) whereas others can be more effective in the therapy of more long-lasting ischemia (reducing Volm), then the analysis described in this study is capable of producing all information required to make a more educated decision in planning the clinical application of an effective drug. For instance, in the case of CNS-1102, the drug may be particularly useful if started very early (ie, before hospital or emergency room) in prolonging the interval that the brain can withstand a thromboembolic event before spontaneous or therapeutic reperfusion. This might increase the efficacy of reperfusion therapy as well as allow a greater number of patients to reach the hospital and qualify for such therapy before substantial damage occurs.

Acknowledgments

This study was supported by grant NS-23979 from the National Institutes of Health, National Institute of Neurolog-
tistical Disorders and Stroke (Dr Grotta). CNS-1102 was supplied by Cambridge Neuroscience, Cambridge, Mass.

References

Editorial Comment

This study addresses the important issue of ischemic duration as a quantifiable contributor to outcome after stroke. It is well recognized that the severity of an ischemic insult influences the apparent "success" or "failure" of a putative therapeutic agent, as does the dose of the agent and treatment timing selected for study. However, relatively few experimental designs titrate this relation between ischemic time and infarct volume. The accompanying article provides a useful example in a rat reversible focal ischemia model of how a standard pharmacological bioassay technique can be adapted for the task of distinguishing efficacy of neuroprotection over a range of ischemic intensities. The investigators correlate the prolongation of ischemia with infarct volume by using logistic analysis and estimating treatment-induced effects on maximum infarct volume (Vol_max) versus ischemic potency, i.e., the duration producing half-maximal infarct size (T_50). The treatments used (hypothermia and various doses of CNS-1102, an N-methyl-D-aspartate receptor antagonist) are not novel but were apparently selected for likely efficacy in severe focal ischemia. Furthermore, a strain of spontaneously hypertensive rats was used, which may limit generalization of the specific experimental findings. Nevertheless, the analytic approach that emerges from this investigation should be considered a potentially valuable technique in comparing the potency of different neuroprotective agents for graded insults (eg, short versus prolonged ischemic time) and discriminating appropriate clinical utility.

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Stroke. 1994;25:2235-2240
doi: 10.1161/01.STR.25.11.2235
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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