Hyperthermia Masks the Neuroprotective Effects of Tissue Plaminogen Activator

Raza Noor, MS; Chen Xu Wang, MD, PhD; Ashfaq Shuaib, MD, FRCPC

Background and Purpose—Previously, we have shown that hyperthermia significantly increased neuronal damage after ischemic injury in a focal embolic model of stroke in rats. In the present study, we examined the effects of hyperthermia on the efficacy of thrombolytic therapy in this stroke model.

Methods—In part A, efficacy of tissue plaminogen activator (tPA) treatment was examined in normothermic and hyperthermic rats after embolization of preformed clots into middle cerebral artery (MCA). In part B, brain perfusion deficits were assessed in rats after MCA occlusion. In part C, blood–brain barrier (BBB) permeability was examined in rats after MCA occlusion. In part D, we examined the influence of hyperthermia on fibrinolytic activity of tPA in vitro.

Results—Results showed that treatment with tPA significantly reduced infarct volume in normothermic and 38°C hyperthermic rats. When compared with normothermic rats, perfusion deficits in hyperthermic rats were significantly increased at both 3 hours and 6 hours after ischemic injury. Compared with normothermic sham-operated rats, Evans blue dye extravasation was increased in the injured rats with 39°C hyperthermia. In vitro study showed that hyperthermia increased the fibrinolytic activity of tPA.

Conclusions—The present study shows that hyperthermia masks the neuroprotective effects of tPA treatment after ischemic injury and that this may be caused by increased BBB permeability, increased edema, and early progression of ischemic penumbral region to irreversibly damaged tissue as shown by progressively increasing perfusion deficits in hyperthermic rats. (Stroke. 2005;36:665-669.)

Key Words: hyperthermia ■ ischemia ■ middle cerebral artery ■ perfusion

Thromboembolism is involved in 80% to 90% of stroke cases, and the majority of ischemic episodes occur as a result of occlusion of the middle cerebral artery (MCA) or its branches. After an ischemic event, neuronal death evolves in a time-dependent fashion determined by the duration and severity of blood flow. Delayed reperfusion can lead to negative sequelae such as the breakdown of blood–brain barrier (BBB) and the development of hemorrhagic transformation and edema. Because the majority of ischemic strokes are caused by thrombotic or thromboembolic arterial occlusions, therapeutic strategies designed to restore cerebral perfusion hold great promise for these patients. Intravenous infusion of tissue plasminogen activator (tPA) is the only scientifically proven effective therapy for acute ischemic stroke. There is increasing evidence that moderate hyperthermia, when present during or after a period of brain ischemia or trauma, markedly exacerbates the degree of resulting neural injury. Clinical studies also indicated that 50% of all stroke patients have escalated body temperatures within 48 hours of a stroke insult. In a prospective study, body temperature proved to be highly correlated with initial stroke severity, infarct size, mortality, and poor outcome.

In animal models of transient forebrain ischemia, elevations of intra-ischemic brain temperature were reported to enhance and accelerate ischemic injury in vulnerable brain regions and induce damage in structures not ordinarily affected. Other studies in focal cerebral ischemia have shown that hyperthermia is deleterious in focal ischemia and that the effects of otherwise neuroprotective drugs in ischemia may be nullified or become deleterious because of hyperthermia.

We have shown previously that hyperthermia significantly increased neuronal damage after ischemic injury in a focal embolic model of stroke in rats. Hyperthermia during and for 3 hours after embolic occlusion of the MCA significantly increased infarct volume and neurological deficits. Numerous other studies have also demonstrated the deleterious effects of controlled hyperthermia in global and nonthromboembolic models of focal ischemia. However, there is not much data on the effects of hyperthermia on the efficacy of therapeutic thrombolysis. The aim of the present study was to examine the effects of controlled hyperthermia on the efficacy of thrombolytic therapy after ischemic injury in focal embolic model of cerebral ischemia in rats. We also exam-
ined the effects of hyperthermia on BBB permeability and perfusion deficits after focal ischemia to determine whether hyperthermia plays a role in ischemic damage by affecting vascular permeability and reperfusion after cerebral ischemia. Moreover, we also performed an in vitro study examining the effects of hyperthermia on fibrinolytic activity of tPA to determine whether hyperthermia affects ischemic damage by changing tPA activity.

Materials and Methods

Stroke Model
Male Sprague–Dawley rats (Charles River, St Constant, Canada) weighing 300 to 350 grams, were used. Animal care and the general protocols for animal use were approved by the Animal Ethics Committee of the University of Alberta. Embolic focal cerebral ischemia was induced by embolizing a preformed clot into the MCA, as described previously.12,21 For the nonthermogenic animals, brain temperature was maintained at 37°C and for the hyperthermic animals it was maintained at 38°C or 39°C before embolizing a clot into the MCA, throughout the surgery, and for 3 hours after ischemic injury as described previously.12

Neurological Deficits and Seizure Activity
Neurological deficits and seizure activity were recorded at 24 hours after ischemic injury. Neurological deficits were determined using a modified Bederson scoring system.4,21 Seizure activity was classified with the Racine scale.14,21 Mortality was also recorded.

Measurement of Infarct Size and Brain Edema
The procedures for assessment of infarct volume have been detailed previously.19,21 The infarct volume was expressed as a percentage of the total volume from the ipsilateral hemisphere. Brain edema was determined by calculating the volume difference between the 2 hemispheres and dividing by the volume of the left hemisphere.17

Detection of Perfusion Deficits
Perfusion deficits were analyzed as described previously.19,20 The perfused microvessels in the brain were visualized using fluorescent microscopy in 9 sections taken serially and 1 mm apart starting at 3.70 mm anterior to the bregma. Areas of perfusion deficits in the cortex and striatum were traced, calculated, and expressed in mm².

Evaluation of BBB Permeability
BBB permeability was evaluated by fluorescent detection of extravasated Evans blue dye.2 Briefly, 2% Evans blue dye in saline was injected intravenously at 24 hours after MCA occlusion and allowed to circulate for 15 minutes. Rats were anesthetized and transcardially perfused with saline until colorless perfusion fluid was obtained from the right atrium. After decapitation, central and peripheral ischemic cortex regions were collected along with the striatum. Evans blue dye content was evaluated by a microplate fluorescence reader at an excitation wavelength of 620 nm and emission wavelength of 680 nm. The amount of extravasated Evans blue dye in each group was determined by calculating the volume difference between the 2 hemispheres and dividing by the volume of the left hemisphere.17

Influence of hyperthermia on fibrinolysis with tPA

Hyperthermic Fibrinolysis With tPA
Influence of hyperthermia on fibrinolysis with tPA was examined by incubating a preformed clot at different temperatures in the presence or absence of tPA. The clots were formed as described previously.12,21 and incubated in 2.5 mL normal blood plasma with 2.5 mL tPA reconstituted with saline and equivalent to 10 mg/kg. In the control groups, 2.5 mL saline was added instead of tPA. Progressive clot lysis was measured every hour and data were expressed as percentage of original length of the clots.

Experimental Design
The present study consisted of four parts. In part A, we examined whether hyperthermia affects neuroprotective actions of tPA. Animals were randomly assigned to the following groups: normothermic (37°C) + saline (n=8); normothermic + tPA (n=8); 38°C hyperthermia + saline (n=8); 38°C hyperthermia + tPA (n=8); 39°C hyperthermia + saline (n=8); and 39°C hyperthermia + tPA (n=8). Saline or tPA (10 mg/kg) was infused intravenously via the tail vein (one-third bolus; two-thirds infused over 30 minutes), and the administration was started at 30 minutes after MCA occlusion. The animals were euthanized at 24 hours after embolization. Because of premature mortality, a total of 67 animals were used to obtain a total of 8 animals in each group for assessment of infarct volume and edema. In part B, we examined whether hyperthermia increases perfusion deficits after ischemic injury. Because of premature mortality, 29 animals were used to obtain a total of 6 animals in each group for assessment of perfusion deficits. The animals were randomly assigned to the following groups: normothermic, euthanized at 3 hours after MCA occlusion (n=6); normothermic, euthanized at 6 hours (n=6); 39°C hyperthermia, euthanized at 3 hours (n=6); and 39°C hyperthermia, euthanized at 6 hours (n=6). In part C, we examined the effects of hyperthermia on BBB permeability after ischemic injury. The animals were randomly assigned to the following groups: normothermic sham-operated (n=3); normothermic-injured (n=6); 38°C hyperthermia sham-operated (n=3); 38°C hyperthermia-injured (n=6); 39°C hyperthermia sham-operated; and 39°C hyperthermia-injured (n=6). In part D, we examined the effects of hyperthermia on fibrinolytic activity of tPA in vitro. Preformed clots were assigned to the following groups: 37°C + saline (n=6); 37°C + tPA (n=6); 38°C + saline (n=6); 38°C + tPA (n=6); 39°C + saline (n=6); and 39°C + tPA (n=6).

Statistical Analysis
The differences in infarct volume, edema, perfusion deficits, BBB permeability, and fibrinolysis were analyzed with 1-way ANOVA. Neurological deficit and seizure scores were analyzed with the Kruskal–Wallis test. Mortality rates were compared with the χ² test. Differences were considered significant when P<0.05.

Results

Part A: Hyperthermia Masks the Neuroprotective Effects of Thrombolytic Therapy With tPA

Infarct Volume
Emboliing a preformed clot resulted in an infarction in the territory irrigated by the MCA. At 24 hours after the MCA occlusion, average infarct volume in the normothermic group was 35.6 ± 2.8% (mean ± SEM) of the ipsilateral hemisphere. In the hyperthermic animals, the infarct volume was significantly increased to 50.9 ± 3.7% (P<0.001) in the 38°C group and to 62.4 ± 2.7% (P<0.001) in the 39°C group. Infarct volume in the 39°C group was also significantly larger than that in the 38°C group (P<0.001). After tPA treatment (Figure 1), the infarct volume in the normothermic and 38°C groups was significantly decreased to 15.7 ± 2.6% and 36.2 ± 3.7% (P<0.001), but not in the 39°C group.

Normothermic animals exhibited an average brain edema of 10.7 ± 2.2%, whereas in the 38°C and 39°C groups, brain edema was increased to 19.2 ± 1.9% and 17.8 ± 2.0%, respectively (Figure 2). Brain edema was significantly increased in the 38°C group only as compared with the normothermic group (P<0.05).

Behavioral Tests
Neurological deficit scores are shown in Table 1. The scores of neurological deficits were significantly higher in both
38°C and 39°C hyperthermia groups when compared with the normothermic animals (P<0.05). Treatment with tPA improved neurological deficits in the 38°C hyperthermia group only (P<0.05).

Seizure activity scores are shown in Table 2. The seizure scores were significantly increased in the 38°C hyperthermia group only (P<0.05). Treatment with tPA resulted in decreased seizure activity in the 38°C hyperthermia group only (P<0.05).

In both normothermic groups, all rats survived until the end of the experiment. Five rats died prematurely in the saline-treated 38°C group and 5 rats died prematurely in the tPA-treated 38°C group. In the saline-treated 39°C group, 3 rats died prematurely and 5 rats died prematurely in the tPA-treated 39°C group. Hyperthermia significantly increased mortality in both 38°C and 39°C groups as compared with control (P<0.05).

Table 1. Neurological Deficits After Focal Cerebral Ischemia

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normothermic (37°C)</th>
<th>Hyperthermic (38°C)</th>
<th>Hyperthermic (39°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.5 (1.5–3)</td>
<td>3 (3–4)*</td>
<td>3.5 (3–4)*</td>
</tr>
<tr>
<td>tPA</td>
<td>2.5 (1–3)</td>
<td>2.5 (2–3)†</td>
<td>4 (3–4)</td>
</tr>
</tbody>
</table>

The neurological deficits were recorded at 24 hours after embolizing a preformed clot into the MCA. Neurological deficit scores are expressed as medians and interquartile ranges; 25th to 75th percentiles are shown in parentheses.

*Significantly higher when compared to the normothermic animals (P<0.05).
†tPA treatment significantly improved neurological deficits when compared to the saline group (P<0.05).

Part B: Hyperthermia Increases Perfusion Deficits After Ischemic Injury

Next, we studied the mechanisms of the deleterious actions of hyperthermia in the ischemic brain injury. Previously, we have shown that perfusion deficits reduced over time after MCA occlusion.20 To determine whether the deleterious effects of hyperthermia are caused by delay in reduction of perfusion deficits, we compared the dynamic changes in perfusion deficits between normothermic and hyperthermic rats. Perfusion deficits were observed in the areas supplied by the MCA after the artery was occluded. The shape of the perfusion deficit was irregular, but the boundary between the perfused and nonperfused regions was usually very clear (Figure 3).

Pooled data from the different groups are shown in Figure 4. In the normothermic rats, the perfusion deficits were 0.64±0.33 mm² (mean±SEM) and 1.18±0.49 mm² at 3 hours and 6 hours after the MCA occlusion, respectively. In the hyperthermic rats, the perfusion deficits were 4.01±0.75 mm² and 10.21±2.46 mm² at 3 hours and 6 hours, respectively. Compared with the normothermic rats, perfusion deficits in the hyperthermic rats were significantly increased at 3 hours and 6 hours (P<0.05).

TABLE 2. Seizure Activity After Focal Cerebral Ischemia

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normothermic (37°C)</th>
<th>Hyperthermic (38°C)</th>
<th>Hyperthermic (39°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1 (0.5–2.5)</td>
<td>4 (2.5–5)*</td>
<td>1.5 (1–4.5)</td>
</tr>
<tr>
<td>tPA</td>
<td>0 (0–1)</td>
<td>0.5 (0–1.5)†</td>
<td>2 (1–2)</td>
</tr>
</tbody>
</table>

Seizure activity scores were recorded at 24 hours after embolizing a preformed clot into the MCA. Seizure activity scores are expressed as medians and interquartile ranges; 25th to 75th percentiles are shown in parentheses.

*Significantly higher when compared to normothermic animals (P<0.05).
†Significantly lower when compared to the saline group (P<0.05).
permeability in focal ischemia. The amount of extravagated Evans blue dye in each group was compared with the normothermic sham operated rats and expressed as fold change (Figure 5). Evans blue dye extravasation was significantly increased in both the peripheral ischemic cortex and striatum of the 39°C hyperthermic rats ($P < 0.05$). There was no significant difference in dye extravasation among the sham-operated groups.

**Part D: Hyperthermia Increases Fibrinolytic Activity of tPA**

In an in vitro study, the effect of hyperthermia on fibrinolysis with tPA was examined by incubating a preformed clot at different temperatures in the presence or absence of tPA. In the 39°C + tPA group, the percent of original clot remaining was significantly smaller when compared with the 37°C + tPA group at 1 hour, 2 hours, and 3 hours. In the 38°C + tPA group, the percent of original clot remaining was significantly smaller when compared with the 37°C + tPA group at 2 hours and 3 hours only. There was no significant difference between clot sizes in the control groups at any of the 3 time points (Table 3).

**Discussion**

Previously, we found that hyperthermia significantly worsened the outcome in an embolic model of stroke. In the present study, we examined the efficacy of thrombolytic therapy with tPA in this model under hyperthermic conditions. The present results confirmed our previous findings that hyperthermia markedly exacerbates neuronal damage after focal ischemic injury. Moreover, our data also show that hyperthermia masks the neuroprotective effects of tPA, because treatment with tPA significantly reduced infarct volume in normothermic rats, whereas in moderately hyperthermic rats (38°C) the neuroprotective effects were less pronounced, and no improvement was seen in severely hyperthermic rats (39°C). tPA treatment also did not improve functional recovery, measured with behavioral tests, in the hyperthermic rats. In addition, tPA treatment also failed to decrease mortality in the hyperthermic rats. Thus, these data clearly show that thrombolytic therapy with tPA is not effective in ischemic brain injury in the presence of hyperthermia.

In vitro studies have shown that hyperthermia facilitates tPA-mediated fibrinolysis. In one study, fibrinolytic activity of tPA increased with increasing temperature as indicated by shorter lysis time and higher concentration of D-dimer fibrin degradation product. After addition of tPA, concentration of D-dimer was approximately tripled and time to complete clot lysis was approximately halved from 30°C to 40°C. Our present results also show an increase in fibrinolytic activity of tPA at higher temperatures as indicated by significant decrease in clot size at higher temperatures. These results thus suggest that the increased fibrinolytic activity of tPA during hyperthermia is offset by its deleterious actions and a net
TABLE 3. Hyperthermic Fibrinolysis With tPA

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C+saline, %</td>
<td>91.4±2.1</td>
<td>88.2±2.1</td>
<td>79.8±1.5</td>
</tr>
<tr>
<td>37°C+tPA, %</td>
<td>81.1±1.9</td>
<td>64.9±1.7</td>
<td>48.6±1.9</td>
</tr>
<tr>
<td>38°C+saline, %</td>
<td>90.8±2.5</td>
<td>82.8±1.6</td>
<td>73.5±2.9</td>
</tr>
<tr>
<td>38°C+tPA, %</td>
<td>76.8±2.2</td>
<td>56.2±3.0*</td>
<td>29.5±3.8*</td>
</tr>
<tr>
<td>39°C+saline, %</td>
<td>90.2±1.2</td>
<td>84.8±2.3</td>
<td>72.4±2.5</td>
</tr>
<tr>
<td>39°C+tPA, %</td>
<td>72.8±1.3*</td>
<td>52.2±2.2*</td>
<td>18.1±2.5†</td>
</tr>
</tbody>
</table>

Preformed clots were incubated for 3 hours and clot size was evaluated at 1 hour, 2 hours, and 3 hours. Data are expressed as percent of original clot length remaining at each time point.

*Significantly different compared to the 37°C+tPA group (P<0.05).
†Significantly different compared to the 38°C+tPA group (P<0.05).

The effect of hyperthermia in ischemic brain injury is neurodestructive. Because hyperthermia caused severe edema in the ischemic injured brain, it is likely that the downstream microvasculature is compressed, thereby preventing restoration of blood flow, although the occlusion materials may be lysed either spontaneously or artificially with tPA. To test this hypothesis, dynamic changes in perfusion deficits between normothermic and hyperthermic rats were compared. Results showed that hyperthermia significantly increased the perfusion deficits. These results, for the first time to our knowledge, show that hyperthermia contributes to the neurodestructive actions by delaying the re-establishment of perfusion in the ischemic injured brain.

Other mechanisms may also contribute to the detrimental actions of hyperthermia on the ischemic brain injury. Brain temperature modulates ischemia-induced BBB opening with remarkable sensitivity. The extravasation of protein tracers across the barrier is markedly exaggerated by intra-ischemic hyperthermia of 39°C in a global model of ischemia. The present results show increased Evans blue dye extravasation after MCA occlusion in 39°C hyperthermic rats and thus confirm the findings that hyperthermia increases BBB permeability after ischemic injury. tPA is known to potentiate N-methyl-D-aspartate receptor-mediated signaling and neuronal death. tPA enhances N-methyl-D-aspartate–evoked Ca2+ influx, which has been shown to be one of the critical events in excitotoxic neuronal cell death. Thus, an increase in the passage of tPA through the BBB during hyperthermia may explain why hyperthermia masks the beneficial effects of tPA-induced thrombolysis.

In summary, the present study shows that hyperthermia significantly increases infarct volume after ischemic insult in an embolic model of cerebral ischemia. Hyperthermia also reduces the neuroprotective effects of tPA in ischemic brain injury. Moreover, hyperthermia increases BBB permeability and causes a delay in the re-establishment of brain perfusion, which in turn accelerates the progression of ischemic penumbral region to irreversibly damaged tissue. The delay of reperfusion is likely caused by increased edema during hyperthermia. These findings are clinically relevant because data clearly suggest that hyperthermia should be combatted assiduously when thrombolytic therapy with tPA is used for treatment of ischemic stroke patients.

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References

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