Rivaroxaban and Apixaban Reduce Hemorrhagic Transformation After Thrombolysis by Protection of Neurovascular Unit in Rat

Syoichiro Kono, MD; Toru Yamashita, MD, PhD; Kentaro Deguchi, MD; Yoshio Omote, MD; Taijun Yunoki, MD; Kota Sato, MD; Tomoko Kurata, MD; Nozomi Hishikawa, MD, PhD; Koji Abe, MD, PhD

Background and Purpose—This study aimed to assess the risk and benefit of tissue-type plasminogen activator treatment after oral anticoagulation with rivaroxaban or apixaban compared with warfarin or placebo.

Methods—Pretreatment with warfarin (0.2 mg/kg per day), rivaroxaban (2 mg/kg per day), or vehicle (0.5% carboxymethyl cellulose sodium salt) was performed for 7 days. Transient middle cerebral artery occlusion was then induced for 120 minutes, followed by reperfusion with tissue-type plasminogen activator (10 mg/kg per 10 mL). Clinical parameters, including cerebral infarction volume, hemorrhagic volume, and blood coagulation, were examined. Twenty-four hours after reperfusion, markers for the neurovascular unit at the peri-ischemic lesion were immunohistochemically examined in brain sections, and matrix metalloproteinase-9 activity was measured by zymography.

Results—The paraparesis score was significantly improved in the rivaroxaban-pretreated group compared with the warfarin-pretreated group. Intracerebral hemorrhage was observed in the warfarin-pretreated group, and this was reduced in the rivaroxaban and apixaban-pretreated groups compared with the vehicle group. Marked dissociation of astrocyte foot processes and the basal lamina or pericytes was observed in the warfarin-pretreated group, and this was improved in the rivaroxaban- and apixaban-pretreated groups. Furthermore, activation of matrix metalloproteinase-9 in the ipsilateral warfarin-pretreated brain was greatly reduced in rivaroxaban- and apixaban-pretreated rats.

Conclusions—This study shows a lower risk of intracerebral hemorrhage after tissue-type plasminogen activator treatment in rats with ischemic stroke that are pretreated with rivaroxaban and apixaban compared with pretreatment with warfarin. Reducing neurovascular dissociation by rivaroxaban and apixaban compared with warfarin could partly explain a reduction in hemorrhagic complications reported in clinical studies. (Stroke. 2014;45:00-00.)

Key Words: apixaban ■ intracranial hemorrhages ■ pericytes ■ rivaroxaban ■ thrombolytic therapy ■ tissue-type plasminogen activator

Cardioembolic cerebral stroke is the major cause of stroke among elderly people in most developed countries. The number of patients with atrial fibrillation has increased according to the growing proportion of elderly individuals. Warfarin is a vitamin K antagonist, which greatly reduces the risk of stroke. However, the major disadvantages of warfarin are a narrow therapeutic range, drug and food interactions, monitoring requirement, and a risk of bleeding. New oral anticoagulants (NOACs), such as rivaroxaban and apixaban, are direct Xa inhibitors. These NOACs are considered as a major breakthrough for the prevention of stroke in patients with atrial fibrillation because they are noninferior or even superior to warfarin for prevention of stroke and are superior for bleeding side effects without troublesome blood monitoring.1–7

A recombinant tissue-type plasminogen activator (tPA), alteplase, has achieved a great outcome for patients with acute ischemic stroke, especially for cardioembolic embolism because of atrial fibrillation. However, tPA is contraindicated in patients on treatment with warfarin with a prothrombin time (PT)–international normalized ratio >1.7 because of an increased risk of bleeding. However, warfarin-treated patients have a higher incidence of symptomatic intracerebral hemorrhage after tPA treatment, despite a PT–international normalized ratio <1.7, compared with those not taking warfarin.3,4 In contrast, warfarin use is not associated with secondary intracerebral hemorrhage after tPA treatment in patients with acute ischemic stroke with a PT–international normalized ratio <1.7.3–7 In the Japan post-Marketing Alteplase Registration...
Study. Additionally, no guideline is available whether tPA can be used for patients pretreated with rivaroxaban or apixaban <4.5 hours of acute ischemic stroke. Therefore, this study aimed to assess the risk and benefit of tPA treatment under NOACs compared with warfarin or placebo.

Materials and Methods

Experimental Model
Male Wistar rats (SLC, Shizuoka, Japan) that were 11 weeks old (body weight, 240–270 g) were divided into 4 groups: vehicle-treated group (0.5% carboxymethyl cellulose sodium salt; V+tPA; n=12), warfarin-treated group (0.2 mg/kg per day; W+tPA; n=13), rivaroxaban-treated group (2 mg/kg per day; R+tPA; n=9), and apixaban-treated group (10 mg/kg per day; A+tPA; n=10; Figure 1 in the online-only Data Supplement). For each drug, the dose and interval between the last intake of drug and the induction of cerebral ischemia were determined to inhibit clot formation by 70% in the rat venous thromboembolism model. Each drug was administered orally for 7 days, starting from when rats were 11 weeks old. Warfarin and rivaroxaban were administered once a day, and apixaban was administered twice a day. Body weight and blood pressure were measured before the first and the last administrations. Blood (0.9 mL) was drawn from the left femoral vein before and 1 hour after the last administration of each drug. PT, activated PT (aPTT), and the thrombin-antithrombin complex were measured. To measure glucose levels, blood samples were also drawn before transient middle cerebral artery occlusion (tMCAO) and immediately after reperfusion.

After 7 days of administration of the vehicle (12 weeks of age), warfarin, rivaroxaban, or apixaban, the rats were anesthetized with a mixture of nitrous oxide/oxygen/isoflurane (69%:30%:1%) during surgical preparation with an inhalation mask. Body temperature was monitored and maintained at 37±0.3°C by using a heating pad during the surgical procedure. The right MCA was occluded by inserting a 4-0 surgical nylon thread with silicon coating through the common carotid artery as described previously. After 120 minutes of tMCAO, the nylon thread was gently removed to restore blood flow in the MCA territory. The rats were treated with tPA (Grtpa; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan; intravenous bolus, 10 mg/kg per 10 mL). To confirm the intravenous bolus injection of tPA (Grtpa; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan; intravenous bolus, 10 mg/kg per 10 mL). To confirm the effects of tMCAO, regional cerebral blood flow (rCBF) of the right frontoparietal cortex region was measured under general anesthesia before, during tMCAO, and at reperfusion through a burr hole using a laser blood flowmeter (Flo-C1; Omegawave, Tokyo, Japan) as described previously. Twenty-four hours after reperfusion, blood pressure was measured and behavior was analyzed.

For histological examinations, after euthanization, the rats were transcardially perfused with heparinized saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2). The whole brain was removed and immersed in the same fixative for 12 hours at 4°C. After washing with PBS, the tissues were transferred into 10%, 20%, and 30% (wt/vol) sucrose gradients and then embedded in powdered dry ice and stored at −80°C. Coronal brain sections, which were 20 μm thick, were prepared using a cryostat at −18°C and mounted on silane-coated glass slides.

For Western blot analyses and gelatin zymography, a different set of rats (each group; n=5) was treated as above. Twenty-four hours after reperfusion, the rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg) and transcardially perfused with chilled heparin (5 U/mL in PBS; pH 7.2). Brains were removed quickly and divided into ipsilateral–peri-ischemic and contralateral–nonischemic hemispheres. Each hemispheric brain was frozen immediately in dry ice and stored at −80°C until use.

All experimental procedures were approved by the Animal Committee of the Graduate School of Medicine and Dentistry, Okayama University (OKU-2013243).

Behavioral Analysis
Before cerebral ischemia and at 24 hours after reperfusion, the rats were tested for behavioral activity and scored according to our previous report.

Histology and Immunohistochemistry
To determine the area of ischemic lesions, sections were stained with hematoxylin and eosin and examined under a light microscope (Olympus SZX-12; Olympus Optical Co). Sections were cut at 2, 0, –2, –4, and –6 mm from the bregma. The infract area was measured at these 5 sections by counting pixels using Photoshop CS5, and the infract volume was calculated by multiplying the infract area by 2 mm of thickness. To analyze brain hemorrhage, iron staining was performed using an enhanced Perl reaction. Brain sections were incubated with Perl solution (5% potassium ferrocyanide and 5% HCl; 1:1) for 45 minutes, washed in distilled water, and incubated again in 0.5% diaminobenzidine tetrahydrochloride with nickel for 60 minutes as described previously.

For immunohistochemistry, the following primary antibodies were used: rabbit anti-collagen IV antibody (1:200; Novotec), mouse anti–glomerular basement membrane in the GFAP/collagen IV double-labeled sections

Vascular Dissociation Index
We assessed the detachment of astrocyte endfeet from the basement membrane in the GFAP/collagen IV double-labeled sections

Figure 1. Clinical and hemorrhagic complications in the groups. A, The paraparesis score was significantly improved in the rivaroxaban-treated (R+IPA) group compared with the warfarin-treated (W+IPA) group. B, Infarction volume was not different among the 4 groups. C, Intra-cerebral hemorrhage volume was significantly larger in the W+IPA group than in the vehicle-treated (V+IPA), R+IPA, and apixaban-treated (A+IPA) groups.
or from pericytes in the GFAP/PDGFRβ double-labeled sections, as well as the detachment of pericytes from vascular endothelial cells as assessed by PDGFRβ/NAGO. This assessment was achieved by randomly choosing 3 levels of the caudate putamen (1.2, 0.7, and 0.2 mm rostral to the bregma) in each rat, and 4 areas in the ipsilateral peri-infarct cortex in each section. Sections were viewed at x100 magnification with a confocal laser microscope. We confirmed the border between the ischemic core and peri-infarct lesion using cresyl violet staining of adjacent sections according to a previous method. We measured the area between astrocyte endfeet and the basement membrane of each blood vessel, as well as the length of each blood vessel. The area-to-length ratio was then calculated as the vascular dissociation index. In the same manner, the area between astrocyte endfeet and pericytes as well as the area between pericytes and vascular endothelial cells were measured, and the area-to-length ratio was calculated in each blood vessel.

Gelatin Zymography

Gelatin zymography was performed using frozen brain tissue from the cerebral cortex. Frozen brain samples were homogenized in 10x volume lysis buffer (150 mmol/L NaCl, 1% SDS, 0.1% deoxycholic acid, and 50 mmol/L Tris-HCl; pH 7.4) containing protease inhibitors. After centrifugation at 9000g for 15 minutes at 4°C, the supernatant was collected. The total protein concentration of each supernatant was spectrophotometrically determined using the Bradford assay (Ultrospec 3100 Pro; GE Healthcare, Tokyo, Japan). The activity of matrix metalloproteinase-9 (MMP-9) in each sample was measured using a gelatin-zymography kit (Primary Cell, Sapporo, Japan) according to the manufacturer’s instructions. In brief, each sample containing 20 μg protein was diluted with the homogenizing buffer in the kit, mixed with an equal volume of sample buffer, and loaded for electrophoresis for 2 hours. The gels were washed and incubated for 24 hours in incubation buffer at 37°C and then stained with Coomassie blue and scanned. Quantitative densitometric analysis was performed by Image J software.

Western blot analysis was performed to evaluate the expression of MMP-9. The online-only Data Supplement provides further details of the method of Western blotting.

Statistical Analysis

All data are presented as mean±SD. Statistical analyses were performed using 1-factor ANOVA followed by Tukey–Kramer postcomparison test. Differences with a probability value of P<0.05 were considered statistically significant.

Results

Clinical Scores

Mean body weight and systolic and diastolic blood pressure were not significantly different among the 4 groups (Table I in the online-only Data Supplement). rCBF immediately declined to <40% of basal levels during tMCAO. After reperfusion, rCBF quickly recovered almost to basal levels in all of the groups. There was no significant difference in rCBF before tMCAO, during tMCAO, or at reperfusion among the 4 groups (Figure II in the online-only Data Supplement). Although 40.9% of the W+tPA group died, only 25.0% of the R+tPA group and 28.6% of the A+tPA group died by 24 hours after reperfusion (Table I in the online-only Data Supplement). The paraparesis score was significantly improved in the R+tPA group compared with the W+tPA group (2.6±4.6 versus 6.6±1.8; P<0.05; Figure 1A). Infarct volume was not different among the 4 groups (Figure 1B). Intracerebral hemorrhage was observed in the W+tPA group in coronal sections (Figure 2), and this was greatly reduced in the R+tPA and A+tPA groups compared with the V+tPA group (Figure 2). Quantitative intracerebral hemorrhage volume was significantly larger in the W+tPA group than in the V+tPA group (P<0.05). However, the R+tPA and A+tPA groups showed a large reduction in hemorrhage volume (Figure 1C; P<0.05).

Blood Analysis

PT, aPTT, and thrombin–antithrombin complex at baseline were not different among the groups (Figure 3A–3C). One hour after the last administration of each drug, PT was significantly prolonged in the W+tPA and A+tPA groups compared with the V+tPA group (both P<0.01; Figure 3A). The aPTT was not different among the 4 groups (Figure 3B). Thrombin–antithrombin complex was significantly reduced in the W+tPA (P<0.05), R+tPA (P<0.01), and A+tPA (P<0.01) groups compared with the V+tPA group, but there was no difference among the W+tPA, R+tPA, and A+tPA groups. This indicates that the antithrombotic effect was similar in the W+tPA, R+tPA, and A+tPA groups.

Figure 2. Representative photographs of intracerebral hemorrhage. Intracerebral hemorrhage was observed in the warfarin-treated group (W+tPA) group in coronal sections, and this was greatly reduced in the rivaroxaban-treated (R+tPA) and apixaban-treated (A+tPA) groups. V+tPA indicates vehicle-treated.

Figure 3. Blood analysis in the groups. Prothrombin time (PT) was significantly prolonged in the warfarin-treated (W+tPA) and apixaban-treated (A+tPA) groups compared with the vehicle-treated (V+tPA) group (A). The activated PT (aPTT) was not different among the 4 groups (B). Thrombin–antithrombin complex (TAT) was significantly reduced in the W+tPA, rivaroxaban-treated (R+tPA), and A+tPA groups compared with the V+tPA group, but there was no difference among the W+tPA, R+tPA, and A+tPA groups (C). *P<0.05; **P<0.01 vs V+tPA, #P<0.05 vs W+tPA.
Blood glucose levels were not different among the 4 groups before tMCAO and immediately after reperfusion (Table II in the online-only Data Supplement).

**Neurovascular Unit**

In the V+tPA group, little dissociation of the neurovascular unit was found in peri-ischemic lesions (Figure 4A(a)). In contrast, a marked dissociation of the basal lamina (collagen IV) and astrocyte foot processes (GFAP) was observed in peri-ischemic lesions of warfarin-pretreated rats (bar=20 μm). The vascular dissociation index estimates the space between each construction (A(b) and B(b)). **P<0.01 vs vehicle-treated (V+tPA); ##P<0.01 vs warfarin-treated group (W+tPA). A+tPA indicates apixaban-treated; and R+tPA, rivaroxaban-treated.

**Western Blot Analysis**

MMP-9 expression in the ipsilateral cerebral cortex tended to be higher compared with that of the contralateral side. There was no difference in MMP-9 expression among the 4 groups in each side of the cerebral cortex (Figure III in the online-only Data Supplement).

**Zymography**

Gelatin zymography showed that there was no activation and no difference in MMP-9 activity in the contralateral cerebral cortex among the 4 groups (Figure 6A and 6B). In contrast, the ipsilateral cerebral cortex showed significant activation of MMP-9 in the V+tPA and W+tPA groups compared with the contralateral sides (Figure 6B; P<0.01). This ipsilateral activation in the W+tPA group was greatly reduced in the R+tPA and A+tPA groups (Figure 6A and 6B; P<0.05).
In the present study, pretreatment with rivaroxaban and apixaban greatly improved the clinical score (Figure 1A) and intracerebral hemorrhage (Figures 1C and 2) compared with warfarin-pretreated rats after thrombolytic therapy with tPA. In the Rivaroxaban Once daily oral direct factor Xa Inhibition and Prevention of Stroke (ROCKET-AF) study, rivaroxaban was noninferior to warfarin in the prevention of stroke or systemic embolism. Additionally, there was no significant between-group difference in the risk of major bleeding, although intracranial and fatal bleeding occurred less frequently in the rivaroxaban group than in the warfarin group. In the Apixaban for Reduction in Stroke and Other Embolism Trial in Atrial Fibrillation (ARISTOTLE) study, apixaban was superior to warfarin in preventing stroke or systemic embolism, caused less bleeding, and resulted in lower mortality.

There is only 1 case report of rivaroxaban plus tPA combination therapy in patients with acute stroke. In that case, the last dose of rivaroxaban was administered 22 hours before admission, when coagulation tests were normal, including PT, and the patient was discharged without any considerable disability or hemorrhagic complications. Rivaroxaban inhibits factor Xa activity and prolongs PT in a dose-dependent manner. However, changes observed in PT can be small, with a minimal effect at 24 hours after administration because the peak plasma concentration of rivaroxaban is 1 to 4 hours after administration and the plasma half-life is 12 hours. Therefore, a patient with a long time interval from administration of rivaroxaban to tPA and with a normal PT may able to be safely treated with tPA, similar to this previously reported case. There are no reports on patients with acute stroke undergoing thrombolysis while taking apixaban.

Which anticoagulant drugs have a high risk of intracerebral hemorrhage in combination with tPA administration is difficult to determine because there is no common marker to compare antithrombotic effects between warfarin and NOACs. A PT assay was developed for monitoring inhibition of the intrinsic pathway and is sensitive to warfarin. In contrast, direct factor Xa inhibitor only affects 1 enzyme in the coagulation cascade. Rivaroxaban and apixaban have shown a concentration-dependent prolongation of aPTT and PT in previous reports. In the present study, significant prolongation of PT was observed in the W+tPA and A+tPA groups (Figure 3A), but aPTT was not different among the 4 groups (Figure 3B). Thrombin–antithrombin complex was significantly reduced in the W+tPA, R+tPA, and A+tPA groups compared with the V+tPA group (Figure 3C). This finding indicated a similar antithrombotic effect in the W+tPA, R+tPA, and A+tPA groups with each dose for obtaining 70% inhibition of clot formation in the rat venous thromboembolism model.

Various proinflammatory mediators, such as MMPs, thrombin, vascular endothelial growth factor, and bradykinin, are increased in the ischemic brain, accompanied by brain edema, endothelial cell death, disruption of tight junctions, and loss of the basal lamina/extracellular matrix (collagen IV, laminin-1, and fibronectin). These changes could be involved in intracerebral hemorrhage associated with tPA therapy. Several experiments have demonstrated that early inhibition of MMP-9 significantly reduces brain infarct size. We recently reported that MMP-9 activation by warfarin plus tPA was improved in dabigatran-pretreated rats. We also observed a similar finding in rivaroxaban- and apixaban-pretreated rats in the present study (Figure 6).

There were only 5 cases of hemorrhagic transformation observed in this study (Figures 1C, 2, 4 and 5). In the present study, the reduction in rCBF during tMCAO was mild. Therefore, we cannot exclude the possibility that the effects of warfarin, rivaroxaban, and apixaban on tPA-induced hemorrhagic transformation observed in this study may have been influenced by the severity of stroke. We chose the suture model in this study because satisfying the same...
conditions of reperfusion in each group to evaluate neurovascular protection of NOACs was important. However, this model is imperfect, and future studies using a clot approach are warranted.

In summary, the present data suggest a lower risk of intracerebral hemorrhage after tPA treatment in rats with ischemic stroke that are pretreated with rivaroxaban and apixaban compared with warfarin pretreatment. Activation of MMP-9 with warfarin causes a marked dissociation of the neurovascular unit, including pericytes, raising the risk of intracerebral hemorrhage after tPA. This neurovascular dissociation is greatly ameliorated by replacing warfarin with rivaroxaban and apixaban reported in the ROCKET-AF and ARISTOTLE trials.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Rivaroxaban and Apixaban Reduce Hemorrhagic Transformation after Thrombolysis by Protection of the Neurovascular Unit in Rats

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Supplemental Methods

Western blot analysis
For western blot analysis, the same samples as those for zymography were used. To evaluate MMP-9 levels, 8 μg of total protein extract was loaded onto an 8% polyacrylamide gel, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was then transferred to a polyvinylidene fluoride membrane (Millipore). The membrane was incubated in 5% skimmed milk in Tris-buffered saline with 0.2% Tween 20 at room temperature for 2 h, and then probed with primary antibodies overnight at 4°C. The dilution of the primary antibody rabbit anti-MMP-9 was 1:2000 (Abcam). After being washed with PBS, the membranes were probed with an appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, United Kingdom) for 1 h at room temperature. Immunodetection was performed with an enhanced chemiluminescent substrate (Pierce, Rockford, IL). After electrochemiluminescence detection, the membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, 0.7% beta-mercaptoethanol) at 50°C for 15 min and then reprobed with a monoclonal anti-β-tubulin antibody (1:20000; Sigma) as a loading control for protein quantification. The signals were quantified with a Lumino image analyzer (LAS 1000-Mini; Fuji Film, Tokyo, Japan), and quantitative densitometric analysis was performed by Image J. This study aimed to assess the risk and benefit of tPA treatment under oral anticoagulation with rivaroxaban or apixaban compared with warfarin or a placebo control.
Supplemental Figures, Table, and Figure legends

Supplemental Fig. 1
The 4 experimental groups, including the V (vehicle) +tPA group, the W (warfarin) +tPA group, the R (rivaroxaban) +tPA group, and the A (apixaban) +tPA group, are shown. Wistar rats were sacrificed 24 h after 2 h of tMCAO. tMCAO, transient middle cerebral artery occlusion; tPA, tissue plasminogen activator.
Supplemental Fig. II

Regional cerebral blood flow (rCBF) of the right frontoparietal cortex region was measured under general anesthesia before tMCAO, during tMCAO, and at reperfusion through a burr hole using a laser blood flowmeter. In the tMCAO experiments, there was no significant difference in rCBF among the 4 groups at any of the time points.
Supplemental Fig. III

Western blot analysis (A) shows no difference in MMP-9 expression among the 4 groups in each side of the cerebral cortex (A). (B) Densitometric analysis of the western blot.
### Supplemental Table I. Physiological parameters in the 4 experimental groups

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<th>V+tPA (n=12)</th>
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<th>R+tPA (n=9)</th>
<th>A+tPA (n=10)</th>
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<tr>
<td><strong>Body weight (g)</strong></td>
<td>285.1 ± 14.4</td>
<td>286.2 ± 12.0</td>
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<td><strong>Before tMCAO</strong></td>
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<td>Systolic BP (mmHg)</td>
<td>158.6 ± 41.2</td>
<td>151.7 ± 24.4</td>
<td>172.4 ± 23.0</td>
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<td>Diastolic BP (mmHg)</td>
<td>95.1 ± 22.5</td>
<td>92.9 ± 14.8</td>
<td>88.1 ± 13.0</td>
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<td><strong>24 h after tMCAO</strong></td>
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<td>Systolic BP (mmHg)</td>
<td>166.0 ± 28.4</td>
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<td>Diastolic BP (mmHg)</td>
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<td><strong>Survival rate (%)</strong></td>
<td>80.0</td>
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Supplemental Table II. Blood glucose levels in the 4 experimental groups

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<th>R+tPA</th>
<th>A+tPA</th>
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<td>(n=5)</td>
<td>(n=6)</td>
<td>(n=8)</td>
<td>(n=5)</td>
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<tr>
<td>Before tMCAO (mg/dl)</td>
<td>190.4 ± 42.5</td>
<td>180.3 ± 30.3</td>
<td>200.1 ± 6.8</td>
<td>198.3 ± 19.7</td>
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<tr>
<td>At reperfusion (mg/dl)</td>
<td>148.0 ± 17.9</td>
<td>158.0 ± 16.6</td>
<td>160.5 ± 18.2</td>
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