Remote Ischemic Postconditioning May Provide Limited Protection in Rats with Subarachnoid Hemorrhage
Yun-Zhen Wang1,2, Ru-Quan Han1, and Zhi-Yi Zuo1

ABSTRACT

Background: Subarachnoid hemorrhage (SAH) is a major form of hemorrhagic stroke. It is not known whether remote ischemic postconditioning (RIP), a promising method to induce neuroprotection, can improve neurological outcome after SAH.

Methods: SAH was induced by endovascular perforation of a right cerebral artery in adult Sprague-Dawley male rats. RIP was performed 5 minutes later by three cycles of 10 minutes ischemia and 10 minutes reperfusion to the bilateral legs.

Results: SAH reduced neurological scores, induced brain edema and increased blood-brain barrier permeability. RIP did not affect the neurological scores and brain edema but partly inhibited the increase of blood-brain barrier permeability after SAH.

Conclusions: These results suggested that RIP may not significantly induce neuroprotection against SAH.

STROKE is a leading cause of mortality and morbidity. About 87% of all strokes are ischemic and the rest are hemorrhagic in the USA. Intracerebral hemorrhage and subarachnoid hemorrhage (SAH) are the two major forms of hemorrhagic stroke (1). Until now, very limited interventions are available to improve neurological outcome after hemorrhagic stroke.

Remote ischemic conditioning refers to a phenomenon in which application of short episodes of ischemia in limbs induces protection in various organs, such as the heart and brain (2, 3). Applying the short episodes of ischemia to limbs before or after a prolonged and damaging episode of ischemia in the vital organs to induce protection is called remote ischemic preconditioning or postconditioning, respectively. Because of the difficulty to predict the occurrence of ischemia in the organs in most cases, remote ischemic postconditioning (RIP) may have better clinical utility than remote ischemic preconditioning. Since remote ischemic conditioning is easy to apply and may have significant clinical application, for example, in the situation of perioperative stroke, clinical studies have been designed and shown that remote ischemic conditioning is safe and well-tolerated even in patients with SAH (4-6).

RIP has been shown to improve neurological outcome after ischemic stroke in animals (7). Remote ischemic conditioning may reduce brain tissue injury in humans with brain trauma or ischemic stroke treated with thrombolysis (8, 9). However, RIP did not provide neuroprotection in rats with intracerebral hemorrhage (10). It is not known whether RIP induced neuroprotection against SAH, the other major form of hemorrhagic stroke. We designed this study to test the hypothesis that RIP improved neurological outcome after SAH.
MATERIALS AND METHODS

The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA, USA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health publications number 80-23, revised in 2011). Efforts were made to minimize the number of animals used and suffering of animals. Our manuscript was written up in accordance with the Animal Research: Reporting In Vivo Experiments.

Experimental Animals and Groups

Two-month old Sprague-Dawley male rats weighing from 300 g to 370 g (Charles River Laboratories Inc., Wilmington, MA, USA) were randomly assigned to three groups: sham-operated (N=16), SAH (N=18) and SAH plus RIP (N=18).

RIP Intervention

Five minutes after SAH establishment, RIP was induced by blocking and releasing the bilateral femoral arteries using a reversible snare, which was formed by passing two ends of a cut rubber band through a small rubber tube. The bilateral femoral arteries were blocked for 10 minutes and then opened for 10 minutes. This ischemia and reperfusion process was performed for 3 cycles as described before (10, 11). During this process, the blood flow in the legs was monitored by a pulse oximeter placed on the bilateral dorsalis pedis arteries. Limb ischemia was confirmed by the disappearance of pulses and the appearance of cyanosis. Reperfusion was confirmed by returning to the original pink color and artery pulsation in the legs. Rats were under anesthesia until the last episode of ischemia was completed (~50 minutes after SAH establishment). Animals in SAH and sham groups received 50 minutes of anesthesia immediately after the surgery to establish SAH.

SAH Establishment

As previously described (12), endovascular perforation was used to induce SAH. Briefly, rats were anesthetized with 2% isoflurane in oxygen, intubated and mechanically ventilated to maintain end-tidal CO₂ between 35 to 40 mm Hg. Their temperature was maintained at 37°C by placing them on a heat pad (Physitemp Instruments Inc., Clifton, NJ, USA). A 4-0 sharp monofilament nylon suture (Beijing Cinontech Co. Ltd, Beijing, China) was advanced into the external carotid artery and then the right internal carotid artery. After resistance was felt, the suture was pushed 5 mm further to perforate the bifurcation of the anterior and middle cerebral artery. The suture was removed to let the blood flow in the internal carotid artery to produce SAH. Sham-operated rats had the same procedure except that the suture was withdrawn after feeling resistance. The heart rates and pulse oximeter oxygen saturations of rats were monitored continuously and noninvasively using a MouseOX Murine Plus Oximeter System (Starr Life Sciences Corporation, Oakmont, PA, USA). The surgical wound of all animals was infiltrated with 0.25% bupivacaine.

Neurological Scores

An 18-point scoring system described by Garcia et al. (13) was used to evaluate the neurological deficits at 24 and 48 hours after SAH. This modified Garcia assessment had six tests including spontaneous activity, spontaneous movement of four limbs, forepaw outstretching, climbing, body proprioception, and response to whisker stimulation (maximally 3 points to each test with possible total scores from 3 to 18 points). This evaluation was performed by a person who was blinded to the group assignments of animals.

SAH Grade

An 18-point SAH severity grading system described previously (14, 15) was used by a person who was blinded to the group assignments of animals. This evaluation occurred at 48 hours after SAH. The basal cistern was divided into six segments. Each segment was scored from 0 to 3 based on the amount of subarachnoid blood clot. A total score was calculated for each rat by adding the scores from the six segments (0-18 points). Animals receiving a score of less than 8 were excluded from the study.
Brain Water Content Assessment
The degree of brain edema was reflected by brain water content. Forty-eight hours after SAH, brains were quickly harvested and separated into the left and right cerebral hemispheres, cerebellum and brain stem. These brain blocks were weighed (wet weight) and then dried in an oven at 100°C for 72 hours. They were weighed again (dry weight). The percentage of the water content was calculated using the following formula: \((\text{wet weight - dry weight}/\text{wet weight}) \times 100\%\) (14, 15).

Blood-Brain Barrier (BBB) Permeability Measurement
As described before (16, 17), BBB integrity was evaluated by Evan's blue extravasation. Forty-eight hours after SAH, Evan's blue dye (2% in saline, 5 ml/kg; Sigma Aldrich Co., St Louis, MO, USA) was injected into right femoral vein and allowed to circulate for 60 minutes. Rats under isoflurane anesthesia were transcardially perfused with saline to remove the intravascular dye. Their brains were removed and divided into the left and right cerebral hemispheres, cerebellum and brain stem. These brain blocks were weighed and homogenized in 2 ml 50% trichloroacetic acid (Sigma-Aldrich Co., St Louis, MO, USA). The homogenates were incubated overnight at 4°C and centrifuged at 13,000 g for 30 minutes. The amount of Evan's blue in the supernatant was measured spectrophotometrically with absorbance at 620 nm. The results were expressed as \(\mu g/g\) brain tissues.

Statistical Analysis
Parametric data were presented as means ± standard deviation (SD) \((N\geq8)\). Sample size estimate analysis showed that 8 repetitions in each group of a study with 3 groups would detect a minimum difference in means of 50% with an expected SD of residuals at 30% and a desired power of 0.8 at a a level of 0.05 by analysis of variance. The results of brain water content and Evan's blue extravasation were analyzed by one-way analysis of variance followed by Tukey test after the confirmation of normal distribution of the data. Neurological scores and SAH grades were analyzed by one-way analysis of variance on ranks followed by Tukey test. A P<0.05 was accepted as significant. All statistical analyses were performed using the SigmaStat program (SYSTAT Software Inc., Point Richmond, CA, USA).

RESULTS
Four rats (two from SAH group and the other two from SAH plus RIP group) were excluded from the study because their SAH grades were < 8. Four animals in the SAH group and 3 animals in the SAH plus RIP group died within 48 hours after SAH. These animals did not contribute data to the final analysis of neurological scores, brain edema and BBB permeability.

The SAH grades were similar between the SAH group and SAH plus RIP group. The neurological scores between these two groups were similar no matter whether the scores were assessed at 24 or 48 hours after the onset of SAH. The neurological scores of these two groups were worse than those of sham group (Figure 1). SAH caused by puncturing a right cerebral artery induced significant edema in the right cerebral hemisphere (water accounted for 78.6 ± 0.4% and 80.0±1.2% of brain tissue wet weight...
for sham group and SAH group, respectively; P<0.05). This edema was not affected by RIP (water accounted for 80.2±1.8% of brain tissue wet weight for SAH plus RIP group; P>0.05 compared with SAH group). There was no significant brain edema in the left cerebral hemisphere, brain stem and cerebellum (Figure 2). The permeability of BBB as assessed by Even's blue content in the brain tissues was increased in all four brain regions in rats with SAH. This increase was partly reduced by RIP (Figure 3).

**DISCUSSION**

We clearly caused SAH by perforating a right cerebral artery in rats. This SAH reduced neurological scores, induced brain edema and increased BBB permeability. However, RIP did not affect the neurological scores and brain edema after SAH, suggesting that RIP under current experimental conditions does not provide neuroprotection against SAH. We used three cycles of 10-minute ischemia and 10-minute reperfusion to postcondition the animals here. This postconditioning method clearly provided neuroprotection in rats with ischemic stroke (7). However, the same method of postconditioning failed to provide neuroprotection against intracerebral hemorrhage (10). This previous finding and our results indicated that RIP may not be an effective method to provide neuroprotection against hemorrhagic stroke.

Nevertheless, although a previous study showed that RIP did not affect the increased BBB permeability after intracerebral hemorrhage (10), our results indicated that RIP may reduce SAH-induced increase of BBB permeability. The reason for this different finding may be due to different hemorrhagic stroke models used in the studies.

Interestingly, RIP partly inhibited SAH-induced increase of BBB permeability but did not affect SAH-induced brain edema in our study. This result suggested that brain edema after SAH is not purely caused by increased BBB permeability. Consistent with this suggestion, our study showed that BBB permeability was increased in all brain regions examined; whereas significant brain edema occurred only in right cerebral hemisphere where arterial perforation happened. In addition, it is known that multiple mechanisms including electrolyte imbalance and hypertension contribute to brain edema after SAH (18).

RIP induces neuroprotection against ischemic stroke (7) but does not appear to provide signifi-
Remote Ischemic Postconditioning and SAH

Yun-Zhen Wang et al.

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Zhi-Yi Zuo conceived the project, Yun-Zhen Wang, Rü-Quan Han and Zhi-Yi Zuo designed the study, Yun-Zhen Wang performed the experiments. Yun-Zhen Wang did the initial data analysis and drafted methods section. Zhi-Yi Zuo performed the final data analysis and wrote the manuscript.

References