

Ginsenoside Rd in Experimental Stroke: Superior Neuroprotective Efficacy with a Wide Therapeutic Window

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Abstract Ginsenoside Rd (Rd), one of the main active ingredients in *Panax ginseng*, has been demonstrated to protect against ischemic cerebral damage *in vitro* and *in vivo*. In this study, we aimed to further define the preclinical characteristics of Rd. We show that Rd passes the intact blood-brain barrier and exerts protection in both transient and permanent middle cerebral artery occlusion (MCAO) in rats. In the dose-response study, Rd (10–50 mg/Kg) significantly reduced the infarct volume on postoperative days (PODs) 1, 3, and 7. This protection was associated with an improved neurological outcome for as many as 6 weeks after transient MCAO, as assessed by modified neurological severity score, modified sticky-tape test, and corner test. For comparison, Rd was significantly more effective than edaravone and slightly more effective than *N*-tert-butyl- α -phenylnitron (PBN). In the therapeutic

window study, Rd exhibited remarkable neuroprotection, even when administered for as many as 4 h after the recirculation of transient MCAO or after the onset of permanent MCAO. Furthermore, in female rats or 16-month-old male rats, the salutary effects of Rd were also observed. These findings suggest Rd is a promising neuroprotectant and provide support for future clinical studies to confirm whether Rd is beneficial in ischemic stroke.

Keywords Ginsenoside Rd · Middle cerebral artery occlusion · Neuroprotection · Stroke · Cerebral ischemia

Introduction

Ischemic stroke ranks among the leading causes of death and adult disability worldwide. Two major approaches have been developed to treat acute ischemic stroke: recanalization and neuroprotection. Thrombolysis with tissue plasminogen activator, limited by its narrow therapeutic time window and the concern of hemorrhagic complication, is still uncommon in use [1]. In the US, only 3 to 5% of patients with ischemic stroke receive tissue plasminogen activator [2]. The goal of neuroprotective strategies is to protect the penumbra brain tissue and attenuate clinical sequelae of stroke. The development of neuroprotective therapy for acute ischemic stroke is challenging. Although a large number of neuroprotective interventions have demonstrated efficacy in preclinical studies involving small animals, clinical studies have so far failed to demonstrate similar efficacy [3, 4].

Ginseng, the root of *Panax ginseng* C. A. Meyer (Araliaceae), has been used as a tonic to treat a wide variety of disorders in China for millennia. Extensive studies have proved that the molecular components respon-

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sible for the pharmacologic effects of ginseng are ginseng saponins, namely ginsenosides. Currently, more than 30 ginsenosides have been identified and isolated from ginseng. Dammar-24(25)-ene-3b,12b,20(S)-triol-(20-O-b-D-glucopyranosyl)-3-O-b-D-glucopyranosyl-(1→2)-b-D-glucopyranoside (ginsenoside Rd, Rd) is one of the main active components of ginsenosides. Previously, we found that Rd could protect against neuronal oxidative damage induced by hydrogen peroxide [5] and oxygen-glucose deprivation [6], an *in vitro* model of cerebral ischemia. More recently, Rd was reported to ameliorate histological and functional outcome after focal cerebral ischemia [7–9], which is associated with mitochondrial protection and energy restoration. However, the preclinical characteristics of the neuroprotective effects of Rd have not been fully defined. Thus, in this study, we sought to characterize the effects of different Rd treatment regimes on the outcome of permanent and transient middle cerebral artery (MCA) occlusion in rats. In particular, we examined the dose response and therapeutic window of the protective effects, and we investigated whether this protection also occurs in female rats or aged rats.

Methods

Focal Cerebral Ischemia

Animal procedures were in accordance with the National Institutes (NIH) of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). All efforts were made to minimize animal number and their suffering. Focal cerebral ischemia was induced by transient or permanent MCAO in Sprague-Dawley rats as described earlier [10]. Briefly, animals were anesthetized with a mixture of isoflurane (1.5–2%), oxygen, and nitrogen. A 4–0 nylon monofilament coated with poly-L-lysine was introduced through the internal carotid artery to occlude the origin of the MCA. We maintained the temperature ($37.0 \pm 0.5^\circ\text{C}$) using a thermostatically controlled heating blanket connected to a thermometer probe in the rectum, and at the same time we monitored physiological parameters, including blood gases (pCO_2 and pO_2), pH, glucose level, and blood pressure. Blood gas was immediately analyzed using a Rapidlab 1260 system (Siemens, Erlangen, Germany). The induction of focal cerebral ischemia was verified with laser Doppler flowmetry (PeriFlux 5000; Perimed AB, Sweden), with the probe placed 6-mm lateral and 2-mm posterior to the bregma [11, 12] and secured with α -cyanoacrylate adhesive. A drop in regional cerebral blood flow (CBF) below 30% of baseline after the insertion of the filament was considered to be sufficient for induction of focal cerebral ischemia. Occlusion of the right

MCA was induced either permanently for 24 h or transiently for 2 h with a reperfusion period of up to 42 days. Control animals were subjected to the same surgical procedures, except that the suture was not advanced into the MCA.

BBB Passage

Rd and blood-brain barrier (BBB)-impermeable bovine serum albumin (BSA) (control) were radiolabeled with tritium and purified as previously described [13]. The radiolabeled reagents (50 mg/Kg) were injected via the tail vein of nonischemic male and female rats. After 0.5, 2, 8, and 24 h after injection of substances, animals were terminally anesthetized with chloral hydrate and perfused carefully with saline, and the whole brain was dissected, blotted dry, and weighed. Blood was centrifuged to obtain the serum. The radioactivity was measured with a liquid scintillation counter.

Administration of Rd

Rd with a purity of 98% was obtained from Tai-He Biopharmaceutical Co. Ltd. (Guangzhou, China). Rd stock solutions were prepared in saline containing 10% 1,3-propanediol (v/v). In dose-response studies, Rd was administered at concentrations ranging from 1 to 50 mg/kg. For comparison, we treated rats with PBN (*N-tert-butyl-alpha-phenylnitron*; Sigma, St. Louis, MO, USA) and edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; Merck, Darmstadt, Germany) using their most effective concentrations. All treatments or vehicle were applied intraperitoneally bolus 30 minutes before MCAO randomly.

The therapeutic window of Rd was investigated with a dose of 50 mg/kg. For transient ischemia, a bolus of Rd was administered at 0, 2, 4, or 8 h after the reperfusion. For permanent ischemia, a bolus injection of Rd was given at 0, 2, 4, or 8 h after the onset of artery occlusion. Vehicle of a similar volume was injected 2 h after MCAO. Rats were assigned to each experimental group randomly before MCAO.

Infarct Size Assessment

On PODs 1, 3, and 7, animals were decapitated and 2-mm thick coronal sections from throughout the brain were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Amresco Inc., Solon, OH, USA) to evaluate the infarct volume. To compensate for the effect of brain edema, the corrected infarct volume was calculated as previously described: corrected infarct area = contralateral hemisphere area (nonlesioned ipsilateral area) [14]. Infarct volume was manually quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA) in a blinded way and expressed as a percentage of the contralateral structure.

Measurement of Edema

At 24 h after stroke, brain edema was measured by comparing the wet-to-dry tissue weight ratios as described earlier [15]. Briefly, the brain was quickly removed after the animal was killed. Then the brain was blotted to remove residual absorbent moisture and dissected through the interhemispheric fissure into the right and left hemispheres. The water content of both hemispheres was determined as the percent difference in tissue weight before and after drying for 3 days at 100°C.

Behavioral Tests

A battery of behavioral tests, including modified neurological severity scores (mNSS) [16], modified sticky-tape test (MST) [17], and corner test [18], were performed before MCAO and at PODs 1, 3, 7, 14, 21, 28, and 42 by two investigators (X.K. and Q.Y.) who were blinded to the experimental groups. All behavioral tests were conducted in the light phase at approximately the same time each day.

mNSS

mNSS is a composite of motor, sensory, reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). In the severity scores of injury, 1 score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher score, the more severe is the injury. See details in Supplemental Table 1.

MST

To assess the somatosensory dysfunction after cerebral ischemia, we performed the modified adhesive removal (sticky-tape) test. Briefly, a nonremovable tape sleeve is wrapped around the animal's paw as a tactile stimulus. When placed in a testing cage, the rats will use their mouth and to a lesser extent the other forelimb to attempt to remove the labels. The time spent attempting to remove the stimulus was recorded during a 30-second observation period. The test was repeated on the opposite limb until sessions consist of 5 trials per limb each day for a total of 10 calculated ratios. The average ratio of left-to-right performance was calculated as the ratio of interest.

Corner Test

A rat was placed between 2 boards each with a dimension of $60 \times 40 \times 1$ cm³. The edges of the 2 boards were attached at a 30° angle with a small opening along the joint between the 2 boards to encourage entry into the

corner. The rat was placed between the 2 angled boards facing the corner and halfway to the corner. When entering deep into the corner, both sides of the vibrissae were simultaneously stimulated. The rat reared forward and upward, then turned back to face the open end. The nonischemic rats turned either left or right, but the ischemic rats preferentially turned toward the ipsilateral (right) side. The turns in 1 vs the other direction were recorded from 10 trials for each rat. Turning movements that were not part of a rearing movement were not scored.

The validity and reproducibility of these behavioral tests have been tested during the preliminary study (Supplemental Table 2). For reproducibility, the intraclass correlation coefficients between the results from 2 independent examiners (X. K. and Y. Q.) are: 0.987 (MST), 0.998 (mNSS), and 0.936 (corner test), suggesting great reporting consistency between different examiners. For validity, the spearman rank-order correlation coefficients between behavior test score and infarct volume on POD 3 are: -0.81 ($p=0.004$) for MST, 0.697 ($p=0.025$) for mNSS, and 0.666 ($p=0.036$) for the corner test, suggesting a strong correlation between behavioral tests and histological lesion.

Statistical Analysis

All results are presented as mean with standard error of the mean. The behavioral test results were analyzed with repeated-measures of analysis of variance, followed by Tukey *post hoc* test. Female rat MCAO and aged rat MCAO results were analyzed using an unpaired *t* test. Other results were analyzed with one-way analysis of variance followed by Tukey HSD post-test for multiple comparisons. Statistical significance was established at $p < 0.05$.

Results

Physiologic Variables

All animals of this study showed similar values for rectal temperature, mean arterial blood pressure, arterial blood gases, and plasma glucose (Supplemental Table 3). The laser Doppler flowmetry signal showed that regional CBF was reduced equivalently in all groups during ischemia (Supplemental Fig. 1). Rats whose CBF remained $>30\%$ of baseline were excluded from further experiment.

Dose-Response Study

A prerequisite for a direct action of Rd on the brain would be penetration of the BBB. We determined the amount of tritiated Rd in brain and serum at 0.5, 2, 8, and 24 h after intravenous injection in noninjured rats, and calculated the

brain/serum ratios of Rd and albumin as an index of BBB permeability. At every observation point, Rd showed a higher brain/serum ratio, which indicated passage of Rd through the intact BBB (Supplemental Fig. 2).

First, we investigated the dose-response characteristics of the histological and functional improvement induced by Rd. Infarct volumetry was performed on PODs 1, 3, and 7 using TTC staining (Fig. 1a). The reduction in infarct size was observed at 10 mg/kg and was greatest at 50 mg/kg, whereas the 1 mg/kg dose was ineffective (Fig. 1b-d). Total infarct volume corrected for brain swelling was reduced by a mean of 59%, 60% and 63%, respectively on PODs 1, 3 and 7 after the treatment with 50 mg/kg Rd. Subanalysis showed that both cortex and striatum were significantly protected (by 57-67% and 46-55%, respectively). Percentage of brain swelling was dramatically diminished by approximately 70% after treatment with 50 mg/kg Rd (Supplemental Fig. 3). For comparison, we tested two other neuroprotectants: edaravone (a reactive oxygen species (ROS) scavenger approved in Japan for the treatment of cerebral infarction) [19] and PBN (an established free

radical spin trap, the parent compound of NXY-059) [20]. Rd was significantly more effective than edaravone, and slightly more effective than PBN in reducing infarct size (Fig. 1b-d).

To determine whether the histological amelioration offered by Rd had consequences for the long-term postischemic behavioral changes, we evaluated neurological deficits using mNSS, MST, and corner test for as much as 6 weeks after transient ischemia (Fig. 2a-c). There were no differences in mortality among all groups (Supplemental Fig. 4). Repeated measures of analysis of variance revealed ischemic rats treated with Rd 50 mg/kg performed significantly better in all test paradigms than vehicle-treated animals, whereas 10 mg/kg dose displayed benefits in MST ($p < 0.001$) and mNSS ($p = 0.049$), but not in the corner test ($p = 0.603$). Rd 50 mg/kg even induced a more robust improvement than edaravone in somatosensory dysfunction, graded according to MST ($p = 0.012$). Moreover, although body weight declined in the first week after ischemia, Rd-treated rats presented a more rapid recovery (Supplemental Fig. 5).

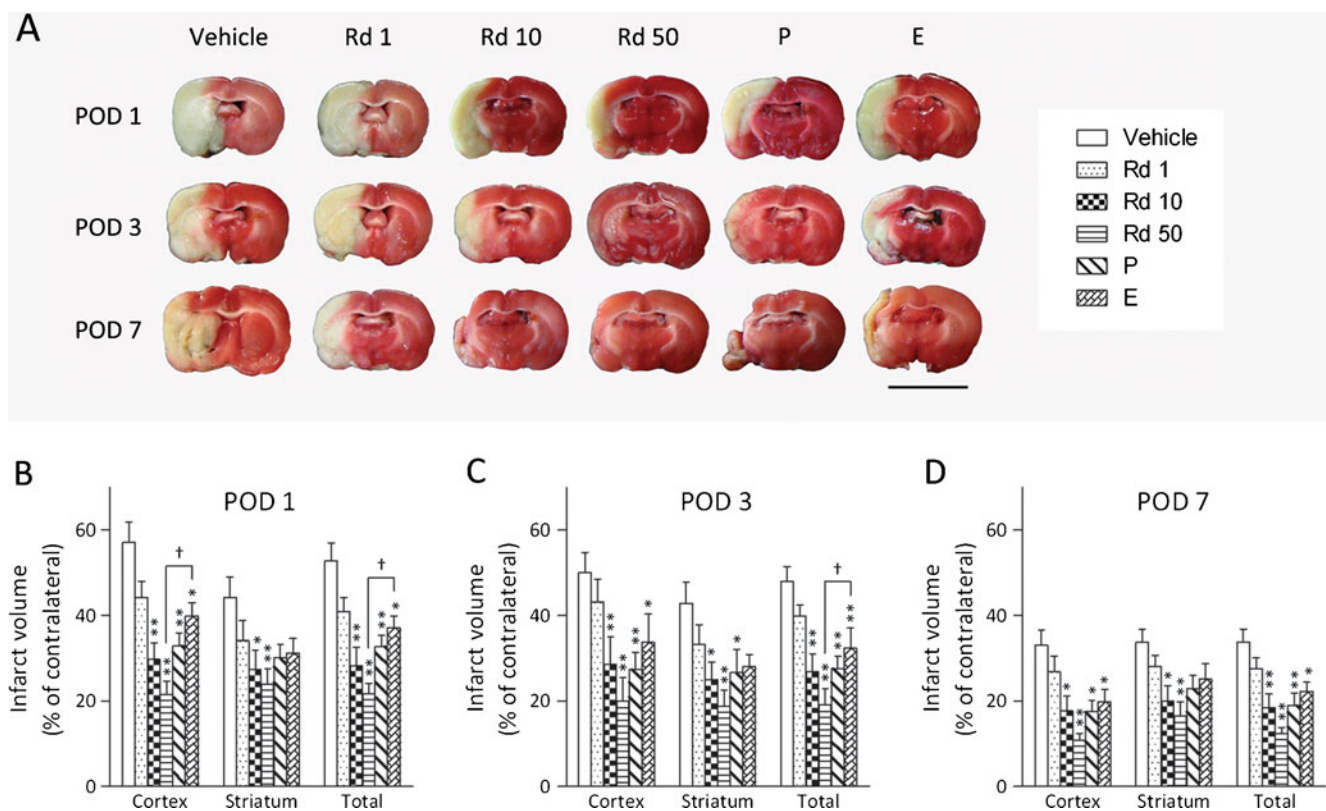


Fig. 1 Dose-response characteristics of the neuroprotection conferred by Ginsenoside Rd (Rd) in transient middle cerebral artery occlusion. **(A)** Representative ischemic lesions at 1.2-mm caudal from bregma in the coronal slices on postoperative days (PODs) 1, 3, and 7. Pale areas represent infarction. Treatment with PBN (P) and edaravone (E). Scale bar, 1 cm. Quantitative analyses of cortical, striatal, and total infarct volumes (corrected for edema) are displayed in **(B-D)**. The infarct size

was significantly reduced in rats pretreated with 10 or 50 mg/kg Rd, as well as with P and E, but not in rats pretreated with 1 mg/kg Rd, as compared with vehicle-treated counterparts. Histograms represent mean \pm standard error of the mean ($n = 8$ to 9 rats for each group). * $p < 0.05$; ** $p < 0.01$ vs vehicle group; † $p < 0.05$ vs edaravone group by 1-way analysis of variance with Tukey post test

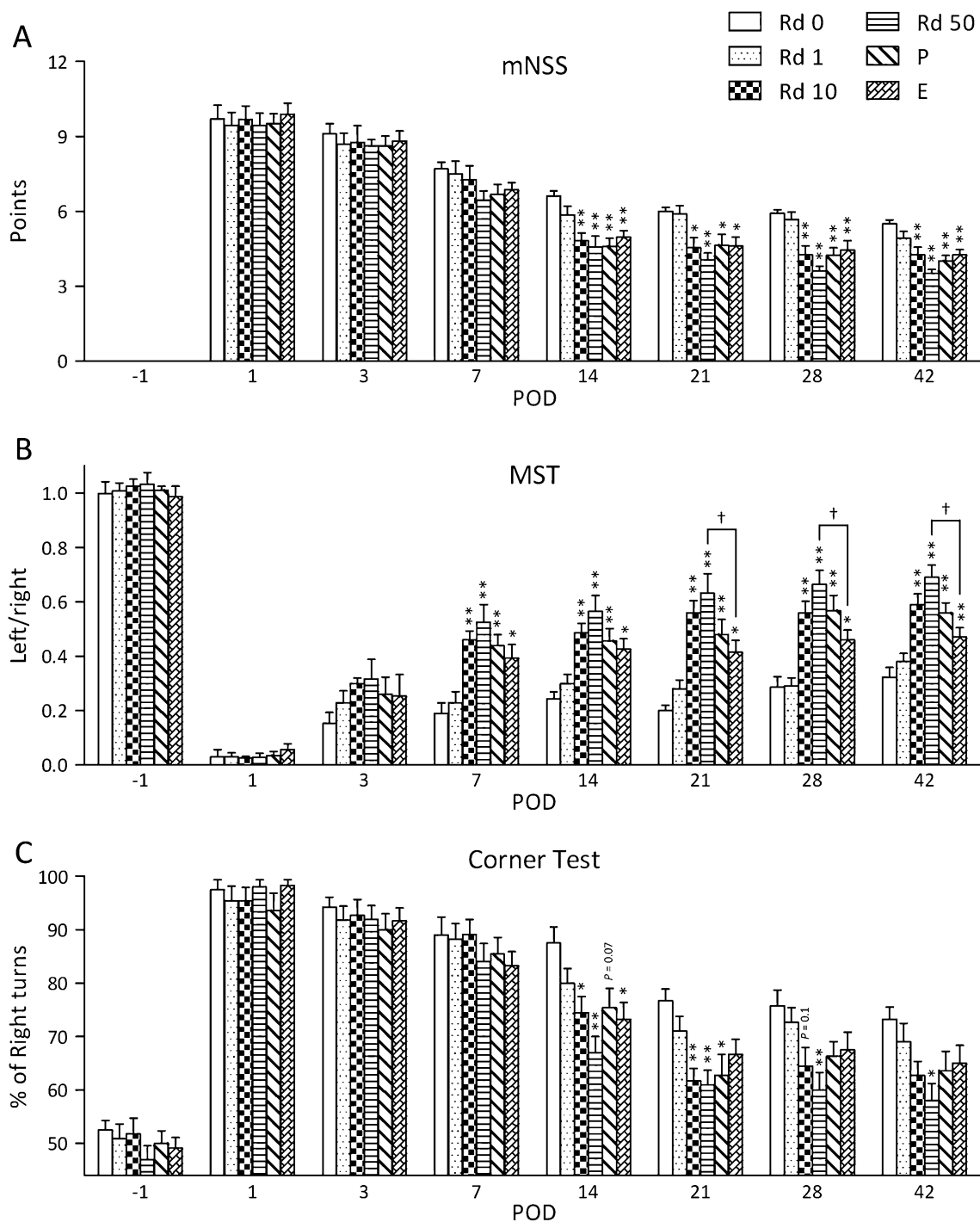


Fig. 2 Dose-response study of neurological deficit after transient middle cerebral artery occlusion. (A) Modified neurological severity scores (mNSS). (B) Modified sticky-tape test (MST). (C) Corner test. Treatment with PBN (P) and edaravone (E). Histograms represent

mean ± standard error of the mean (n=10 to 12 rats for each group). **p*<0.05; ***p*<0.01 vs vehicle group; †*p*<0.05 vs edaravone group by 1-way analysis of variance with Tukey post test. POD = postoperative day; Rd = Ginsenoside Rd

Therapeutic Window Study

Then we sought to define the time interval after transient ischemia at which Rd was still able to protect the brain (50 mg/kg Rd or vehicle was administered at 0, 2, 4, or 8 h

after reperfusion). Significant stroke volume reductions were observed when Rd was administered at 2 h (36-44%) or 4 h (31-40%) after reperfusion, but not when the administration was delayed by 8 h (Fig. 3). Subanalysis showed that both cortex and striatum were significantly

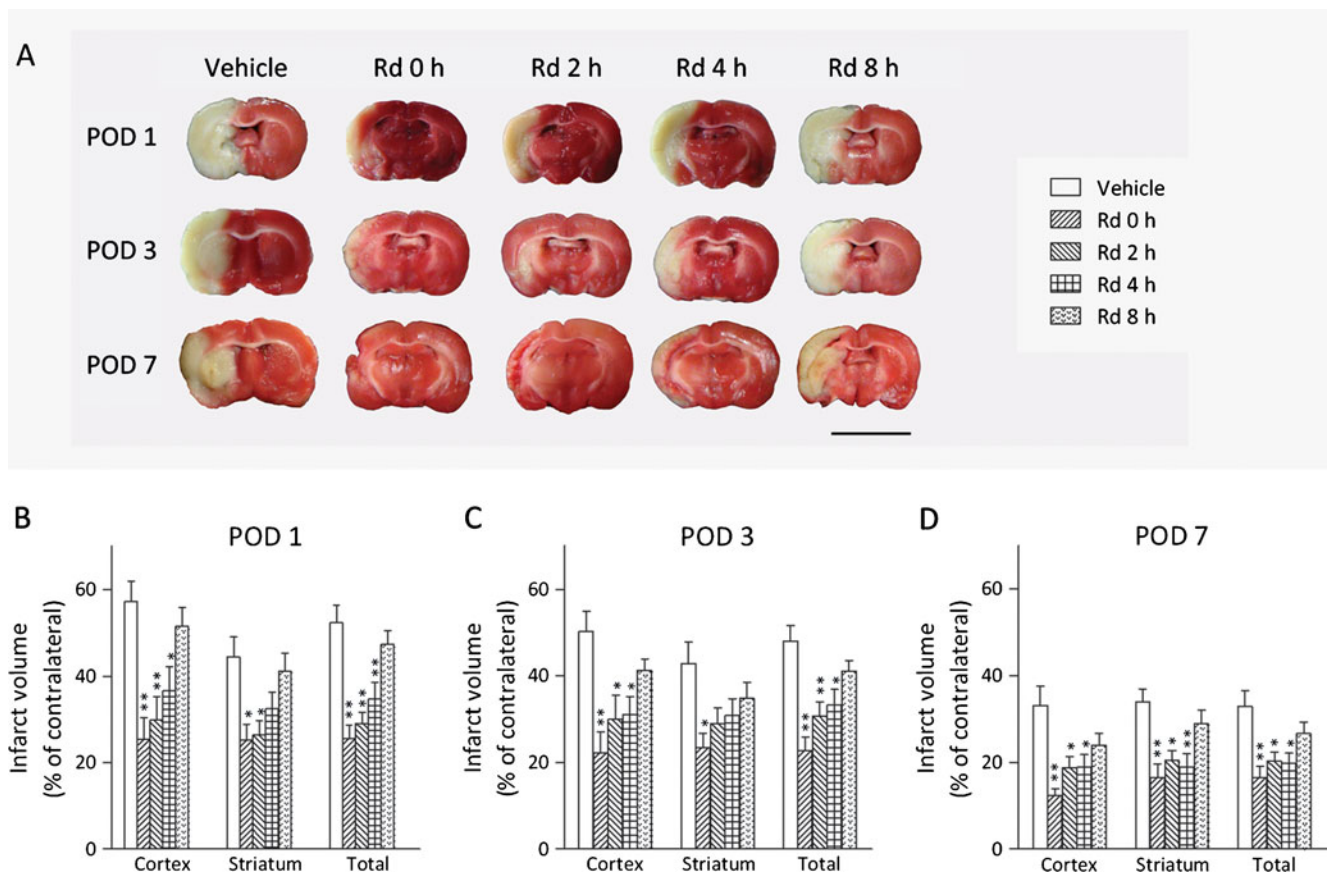


Fig. 3 Therapeutic window characteristics of Ginsenoside Rd (Rd) treatment in transient middle cerebral artery occlusion. (A) Representative ischemic lesions at 1.2-mm caudal from bregma in the coronal slices on postoperative days (PODs) 1, 3, and 7. Scale bar, 1 cm. Quantitative analyses of cortical, striatal, and total infarct volumes (corrected for edema) are displayed in (B–D). The infarct size was

significantly reduced in rats treated with 50 mg/kg Rd 2 h or 4 h after recirculation, but not when Rd administration was delayed up to 8 h after recirculation, as compared with vehicle-treated counterparts. Histograms represent mean \pm standard error of the mean ($n=8$ to 9 rats for each group). * $p<0.05$; ** $p<0.01$ vs vehicle group by 1-way analysis of variance with Tukey post test

protected. Consistently, a significant improvement of neurological outcome was detected when Rd treatment was started 2 or 4 h after reperfusion as graded by mNSS (Fig. 4a), MST (Fig. 4b), and corner test (Fig. 4c).

Permanent Ischemia Study

In another model of cerebral ischemia, permanent MCAO, rats administered with Rd (50 mg/kg) starting at 0, 2, 4, or 8 h after the onset of artery occlusion. Then, 24 hours after commencing vehicle treatment, a greater damage was observed compared with that seen in the transient MCAO (62.6% vs 52.2% of contralateral hemisphere). Treatment with Rd produced a significant reduction in ischemic damage when applied at 0, 2, and 4 h after MCAO, with neuroprotection apparent in both the cortex and the subcortex (Fig. 5). In a further group, Rd was applied starting 8-h postocclusion, whereas the mean volume of total damage was 21% smaller than the vehicle group; this decrease was not statistically significant ($p=0.212$).

Rd is Also Neuroprotective in Female Rats and Aged Rats

Many neuroprotective strategies are not effective in females or in the aged population [21, 22]. As recommended by the Stroke Therapy Academic Industry Roundtable (STAIR), efficacy studies should be further performed in females and aged animals [23, 24]. Therefore, next we sought to determine whether Rd was also neuroprotective in young adult female rats or in 16-month aged male rats. Middle cerebral artery was transiently occluded in subject rats and Rd (50 mg/kg), or the vehicle was administered immediately after reperfusion. Consistent with previous studies [25], female rats had substantially smaller infarcts than male rats (30.7% vs 52.2% of contralateral hemisphere). Figure 6a illustrated that Rd produced a significant reduction in ischemic damage, with an apparent neuroprotection in the cortex (-40% ; $p=0.02$) and a borderline neuroprotection in the subcortical area (-31.6% ; $p=0.06$). However, Rd treatment did not improve the neurological recovery in female rats after MCAO, as measured by mNSS (Fig. 6b). Contrarily, aged rats had

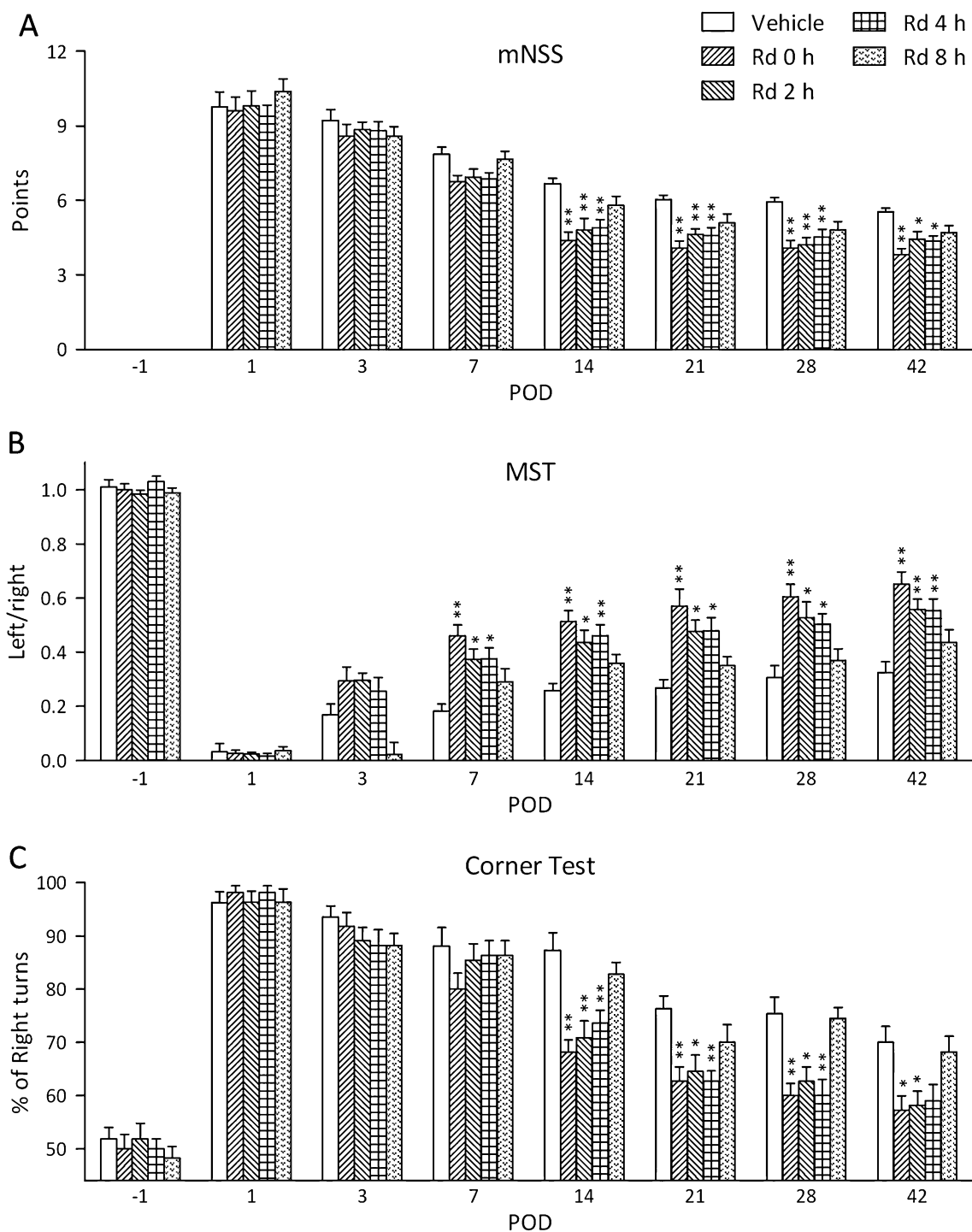


Fig. 4 Therapeutic window study of neurological deficit after transient middle cerebral artery occlusion. **(A)** Modified neurological severity scores (mNSS). **(B)** Modified sticky-tape test (MST). **(C)** Corner test. Histograms represent mean \pm standard error of the mean

($n=11$ to 12 rats for each group). $*p<0.05$; $**p<0.01$ vs vehicle group by 1-way analysis of variance with Tukey post-test. POD = postoperative day; Rd = Ginsenoside Rd

somewhat larger infarcts than young adult rats (58.6% vs 52.2% of contralateral hemisphere). Rd of 50 mg/kg resulted in an infarct volume reduction of 37% ($p=0.005$; Fig. 6c), which is correlated with behavioral improvements as graded by mNSS ($p<0.001$; Fig. 6d).

Discussion

Here we demonstrated a dose-response relationship and defined the therapeutic window for the neuroprotective effect of Rd in transient and permanent cerebral ischemia. The

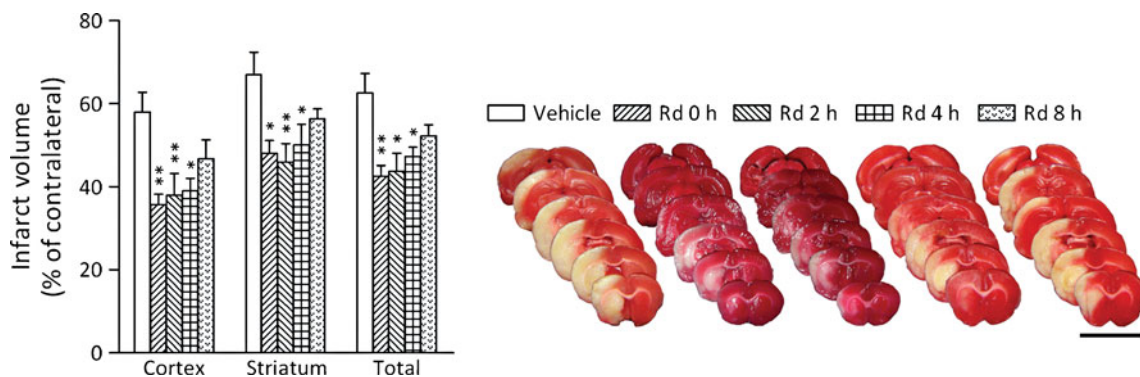


Fig. 5 The effect of delayed administration of Ginsenoside Rd (Rd) on the infarct volume after permanent middle cerebral artery occlusion (MCAO). A bolus injection of 50 mg/kg Rd was applied at 0, 2, 4, and 8 h after the induction of ischemia. Infarct volumetry was performed at 24-h post-MCAO. The infarct size was significantly reduced in rats

treated with Rd 2 h or 4 h after stroke, but not when Rd administration was delayed up to 8 h after MCAO onset, as compared with vehicle-treated counterparts. Histograms represent mean \pm standard error of the mean ($n=8$ to 9 rats for each group). * $p<0.05$; ** $p<0.01$ vs vehicle value using 1-way analysis of variance with Tukey post-test

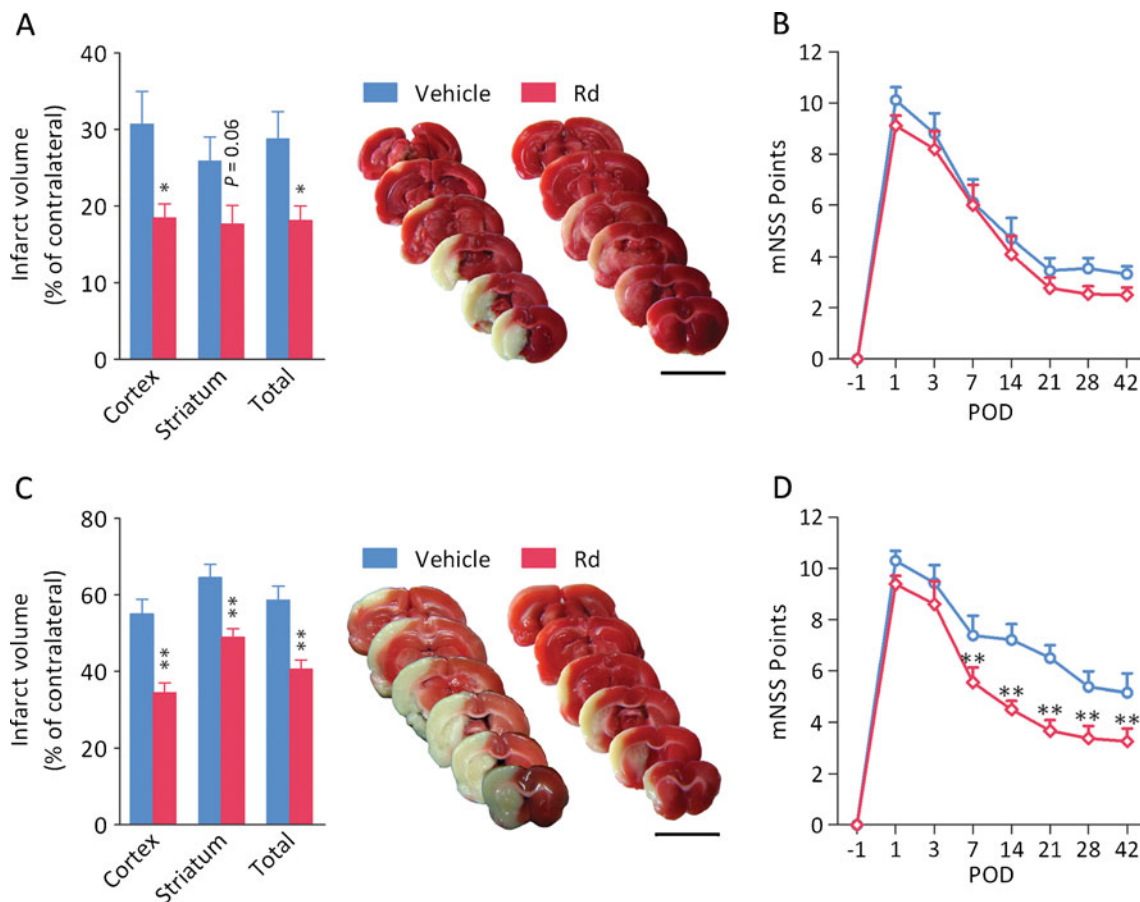


Fig. 6 The neuroprotective effect of Ginsenoside Rd (Rd) is present in female rats and aged rats. Female or aged rats were subjected to 2-h middle cerebral artery occlusion. A bolus injection of 50 mg/Kg Rd was applied immediately after the recirculation. Infarct volumetry was performed on postoperative day (POD) 1. (A) Rd significantly reduced infarct volume in female rats. Scale bar, 1 cm. (B) Rd did not affect the long-term

neurological deficit in female rats as graded by modified neurological severity scores (mNSS). (C) Rd significantly reduced infarct volume in aged rats. Scale bar, 1 cm. (D) Rd ameliorated the long-term neurological deficit graded by mNSS. Data represent mean \pm standard error of the mean ($n=9$ to 12 rats for each group). * $p<0.05$ vs vehicle value by unpaired t test

protective effect of Rd is presented in moderate doses (10–50 mg/kg) and associated with an improvement of the neurological deficits for up to 6 weeks. Importantly, Rd is effective, even if the treatment was delayed to 4 h after recirculation in transient ischemia, or to 4 h after artery occlusion in permanent ischemia. Furthermore, the neuroprotection also occurs in female rats and in aged rats.

In the first part of this study, we showed that the pretreatment of Rd at doses of 10 to 50 mg/kg substantially reduces the infarct size and improves long-term neurological recovery. These observations are consistent with previous reports demonstrating the neuroprotective efficacy of Rd when administered 1 h before ischemia at doses of 5 to 40 mg/kg [9, 26]. However, our infarct volumetry displays more information because of the subanalysis of total infarct volume compared with cortical and striatal infarct volume and because of the infarct volumetry performed on PODs 1, 3, and 7. The spatial pattern of the infarction suggests that Rd is able to salvage jeopardized tissue in both cortical and striatal areas, implying a potential role of Rd for white matter protection. As noted by the STAIR, it is more likely that the treatment targeting both neurons and white matter would have widespread clinical relevance [24]. Striatum is typically more severe than cortex because the blood flow in striatum is lower than in the cortex, and there is little collateral blood supply in deep white matter [27], which supports vital communication between neurons and conveys information among millions of efferent and afferent fibers. Because of its highly packed structure, lesions located in striatum can produce devastating neurological deficits, making it important that Rd significantly attenuates striatal damage.

Stroke is a highly variable clinical condition, and in particular, the delay in spontaneous reperfusion is a variant. Therefore, it is vital that potential therapeutic compounds are able to ameliorate the consequences of permanent occlusion in an animal model [23]. In contrast to transient ischemia, the free radical production was much less prominent after permanent MCAO. However, Rd at the dose of 50 mg/kg, the most effective dose in transient ischemia, still produced a modest effect in a permanent occlusion model (59% reduction of infarct size in transient MCAO vs 37% reduction in permanent MCAO). These results indicate that Rd is active in a model of ischemia without reperfusion, and that this effect, therefore, is not dependent on reperfusion time, which reinforces the interest of Rd in stroke.

Another major observation made in the current investigation was the favorable time window of therapeutic opportunity that is available with Rd administration. Rodent studies appear to be relevant to address a therapeutic window for neuroprotective drugs [24]. In the past, drugs such as the N-methyl-D-aspartate antagonists demonstrated

their efficacy in animals for only a limited after stroke onset; this limitation may have contributed to their lack of efficacy in clinical trials. Here we found the salutary effects of Rd were still present, even when the compound was given 4 h after the recirculation in transient ischemia or 4 h after artery occlusion in permanent ischemia. This means that it is effective in rodents in a time window that is relevant to clinical practice. However, it is worthy to note that in more than 1-half of the completed trials, the enrollment time window was beyond 6 h [3]. Thus, it is advised that the clinical trials of neuroprotection must be designed with a very early window to treatment. To increase the likelihood of determining efficacy, initial clinical studies of neuroprotective agents should not include patients who have received thrombolytic drugs. Potential synergistic or harmful effects when combining with thrombolytics can be explored afterward [4].

The mechanisms of the protective effects of Rd have been recently investigated. We have previously reported that Rd protects against neuronal oxidative damage induced by hydrogen peroxide [5] and oxygen-glucose deprivation [6]. More recently, we suggested that Rd exerts neuroprotection against transient focal ischemia in an integrated process of free radicals scavenging, mitochondrial protection, energy restoration, and apoptosis inhibition [7, 8]. Alternatively, in a model of excitotoxicity, Rd attenuates the Ca^{2+} overload and apoptosis produced by exposure of neuronal cultures to glutamate [28]. This effect is related by the ability of Rd to inhibit Ca^{2+} influx through receptor- and store-operated Ca^{2+} channels without affecting voltage-dependent Ca^{2+} entry [29]. Rd has also been noted to mitigate neuroinflammation [30] and nitric oxide overproduction [31]. Therefore, Rd is neuroprotective in both *in vitro* and *in vivo* models of cerebral ischemia, and could act on multiple pathways of the ischemic cascade, suggesting Rd might have a greater chance of success to impede ischemic injury in acute stroke [4].

Although many compounds with neuroprotective action are in various stages of the pharmaceutical pipeline, Rd represents unique advantages. *Panax ginseng* and its related species have already had thousands of years of human exposure with little reported toxicity. Rd is lipophilic and can easily diffuse across biological membranes and BBBs, a feature that contrasts strongly with NXY-059, which has a very low BBB permeability and negligible cellular penetration [3]. Plus, unlike some perfusion-dependent neuroprotective therapies, such as tirilazad [32], hyperbaric oxygenation [33], and anti-intercellular adhesion molecule-1 antibody [34] are efficacious only in transient cerebral ischemia models. Rd provides substantial neuroprotection in both transient and permanent MCAO models of stroke. Moreover, we herein compared the neuroprotective potency of Rd with other established antioxidants with

neuroprotective abilities. Rd resulted in better histological and functional improvement than edaravone, an ROS scavenger approved in Japan for the treatment of cerebral infarction.

Despite spectacular success of neuroprotective therapy in an animal model of stroke, the translation of neuroprotective benefits from bench to bedside has not been successful. Past failures enhance, not diminish, the need for future neuroprotection research. It is hoped that adherence to the STAIR recommendations may enhance the chances for success [24]. In the present study, all efforts were implemented with the intent to better the study quality, according to STAIR criteria, to ensure the results were reliable and not biased. Currently Rd is being developed to treat patients with acute ischemic stroke. Several studies have been reported demonstrating the pharmacokinetics [35], safety [36], and preliminary efficacy [37] of Rd in human subjects. More recently, a phase III multicenter clinical trial of Rd showed a benefit in terms of the prespecified primary endpoint, the distribution of disability as measured by the modified Rankin score at 90 days after stroke onset. Together, both laboratory and clinical evidence indicate that Rd may be a promising neuroprotectant and provide support for future studies to confirm whether Rd is beneficial in ischemic stroke.

In summary, the present study suggested that Rd treatment reduces both cerebral lesions and functional deficit induced by focal cerebral ischemia. We demonstrated that this effect occurs within a large therapeutic window and is reproducible in different models of stroke (with or without reperfusion) and different populations (male or female, young adult or aged). Therefore, Rd appears to be worthy of clinical development as a treatment for acute ischemic stroke.

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Disclosure The authors declare no conflict of interest.

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