Inhibition of Salvianolic Acid B and Ginsenoside Rg1 on Hemorrhagic Transformation after Stroke

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Abstract

Objective Application of recanalization on stroke patients is not only limited with time window, but also accompanied with the risk of hemorrhagic transformation. In present study, the effects of salvianolic acid B and ginsenoside Rg1 combination (SalB/Rg1) on time window and hemorrhagic transformation against ischemic stroke was evaluated on middle cerebral artery occlusion (MCAO) mice.

Methods The protection and time window of SalB/Rg1 were estimated through infarct volume, neurobehavioral deficits, and histomorphological examination. The prohibition of SalB/Rg1 against hemorrhagic transformation was detected on MCAO mice stimulated with dextrose and reperfusion. Hemorrhagic transformation was assessed by the Heidelberg Bleeding Classification. The mechanism of SalB/Rg1 against hemorrhagic transformation was identified by immunofluorescence staining and in situ gelatin zymography.

Results First, SalB/Rg1 significantly reduced infarct volume and improved neurobehavior in a dose-dependent manner. Then, the protective time window up to 9 hours was detected for SalB/Rg1 against stroke. Both the dose-dependent efficiency and longtime protection of SalB/Rg1 were further identified based on cytoarchitecture through histopathological stain. Second, SalB/Rg1 downregulated hemorrhagic score, infarct volume, and abnormal neurobehavior. Finally, the inhibition of SalB/Rg1 against hemorrhagic transformation was found to accompany with its protection on the integrity of neurovascular unit. Around the edge area of infarction, SalB/Rg1 attenuated the astrocyte activation, maintained the abundance of junction protein (claudin-5) between endothelial cells, considerably decreased matrix metallopeptidase 9 activity through in situ gelatin zymography.

Conclusion SalB/Rg1 is a promising strategy for further development against stroke, especially against hemorrhagic transformation.

Keywords
- stroke
- salvianolic acid B
- ginsenoside Rg1
- hemorrhagic transformation
- neurovascular unit

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Cardiogenic stroke accounts for 11% of all strokes and 25% of ischemic stroke, which always involve occlusion of large vessel such as middle cerebral artery.[1] Compared with those who have other ischemic stroke subtypes, patients with cardiogenic strokes have more serious symptoms, poorer prognoses, or early hemorrhagic conversion after stroke onset. Despite improving survival in recent years, disability of patients with cardiogenic stroke remains high, and there are limited evidence-based therapeutic interventions known to improve patient outcomes clearly.[2,3]

At present, recanalization by intravenous recombinant tissue plasminogen activator (tPA) or by endovascular thrombectomy remains the first-line therapy.[4] Both of them reduce mortality and disability with restoration of blood flow to save ischemic penumbral tissue promptly. While the time windows are up to 4.5 and 6 hours for tPA treatment and endovascular thrombectomy, respectively. Less than 25% of patients benefit from recanalization.[5,6] Disability of patients without recanalization was 41.3% and mortality reached to 16.7% at 30 days from stroke onset.[7] A total of 3 to 8% patients treated with tPA occur hemorrhagic transformation, and 7 to 19% patients treated with endovascular thrombectomy occur hemorrhage.[8] It is urgent need to develop new strategies to extend the time window for recanalization or to reduce hemorrhagic transformation after recanalization.

Traditional Chinese medicine showed prominent advantages in cardiovascular and cerebrovascular aspects.[9] Salvianolic acid B and ginsenoside Rg1 (SalB/Rg1) are the main active components of Danshen (Salviae Miltiorrhizae Radix et Rhizoma) and Sanqi (Notoginseng Radix et Rhizoma), respectively. The combination of SalB/Rg1 showed significant therapeutic effect against myocardial ischemia–reperfusion injury and acute ischemic stroke.[10–12]

In the present study, we further unearth the characteristics of SalB/Rg1 against stroke, such as time window for treatment and efficiency against hemorrhagic transformation.

Methods

Reagents and Materials

SalB and Rg1 were purchased from Shanghai Yousi Bio-Tech Co., Ltd (Shanghai, China). Purity of SalB or Rg1 was $>99\%$, which was evaluated by high-performance liquid chromatography (►**Supplementary Fig. 1**). Zoletil50 was purchased from France Virbac Co., Ltd (Nice, France). Hematoxylin and eosin (H&E) stain solution kit was purchased from Shanghai YiFan Bio-Tech Co., Ltd (Shanghai, China) and Nissl stain solution was purchased from Beyotime Bio-Tech Co., Ltd (Shanghai, China). 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma Aldrich Co., Ltd (St. Louis, Missouri, United States). Novo-light multiple fluorescence immunohistochemical staining kit was purchased from WiSee Biotechnology (Shanghai, China). Glial fibrillary acidic protein (GFAP, Cat# BA0056) antibody and claudin-5 (CLDN-5, Cat# BA3087–1) antibody were purchased from Boster Biological Technology Co., Ltd (Shanghai, China). Dye-quenched (DQ) gelatin was purchased from Invitrogen (Shanghai, China). Other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd unless specifically stated. Filaments were purchased from Pingdingshan Yushun Bio-Tech Co., Ltd (Guangzhou, China).

Establishment of Permanent Middle Cerebral Artery Occlusion Model

Healthy C57BL/6 male mice, weighing 25 to 28 g, were provided by Shanghai SLAC Laboratory Animal Co. and were maintained at temperature-controlled room (22 ± 2° C) with 12-hour cycle of light and dark. Mice were allowed free access to water and diet.

Postanesthesia with 50 mg/kg Zoletil 50 and 10 mg/kg xylazine intraperitoneally, mice were placed in the supine position. Following shaving and sterilizing the neck, the left common carotid artery, external carotid artery, and internal carotid artery were exposed and separated along the inner edge of sternocleidomastoid. Permanent middle cerebral artery occlusion (pMCAO) model was induced by advancing a filament (0622, top diameter 0.22 ± 0.01 mm) up to occlude the origin of the middle cerebral artery for 24 hours. Sham operation was performed using an identical procedure without filament occlusion. To reduce mortality rate, mice were placed in a 35°C incubator (Yuyan, Shanghai) to wake up.

Establishment of Hemorrhagic Transformation Model

Hemorrhagic transformation was induced on middle cerebral artery occlusion (MCAO) mice stimulated with high dextrose and reperfusion. Briefly, 7 mg/kg dextrose was injected intraperitoneally 15 minutes before ischemia and the filament was removed after 3-hour ischemia to restore blood flow for 21 hours. Sham operation was performed using an identical procedure without filament occlusion. To reduce mortality rate, mice were placed in a 35°C incubator to wake up.

SalB/Rg1 Treatment

To evaluate the protection of SalB/Rg1 on pMCAO model, all the mice were randomly assigned into four groups ($n = 8$ for each group). Schematic diagram of the experimental design was shown in ►**Supplementary Fig. 2A**. Sham-operated mice were given saline as normal control (Sham). The other three groups underwent pMCAO surgery, one group of pMCAO mice were given saline as model control (0 mg/kg), another group of pMCAO mice were given 3.125 mg/kg SalB/Rg1 for intervention, and the last group of pMCAO mice were given 6.250 mg/kg SalB/Rg1 for intervention. Intravenous injection of saline or SalB/Rg1 was conducted after the occlusion with filament immediately.

To evaluate the time window of SalB/Rg1 against cerebral ischemia on the pMCAO model, four batches of experiments based on time point of SalB/Rg1 treatment at 3, 6, 9, and 15 hours after MCAO were conducted. Schematic diagram of the experimental design was shown in ►**Supplementary Fig. 2B**. For every time point of SalB/Rg1 treatment, the pMCAO mice were randomly grouped into two groups ($n = 9$ for each group). One group of pMCAO mice were given saline as model control (Saline), another group of pMCAO mice were given 6.250 mg/kg SalB/Rg1.

To evaluate SalB/Rg1 against hemorrhagic transformation after ischemic stroke, the mice were randomly divided into three groups ($n = 16$ for each group). Schematic diagram of the experimental design was shown in ►**Supplementary Fig. 2C**. Sham mice were given saline as normal control (Sham). The other two groups were performed hemorrhagic transformation induction, one group of mice were given saline as model control hemorrhagic transformation (HT), and another group of mice were given 6.250 mg/kg SalB/Rg1. Saline or SalB/Rg1 was administered intravenously immediately after removing filament.

Evaluation of Neurobehavioral Deficits

Neurobehavioral assessments was a quantitative indicator of disease severity and was commonly used as a prognostic indicator after stroke in clinical practice. Therefore, neurobehavioral assessments was not only an important means to reflect neurobehavioral deficits of animals, but also to evaluate whether ischemic stroke model was established. We prescreened all mice and excluded mice with hanging time < 90 seconds and modified neurological severity (mNSS) score > 0. Neurobehavioral deficits were evaluated 24 hours after ischemia by investigators who were blinded to the experimental grouping.

For hanging time test, mice were placed on the cage lid and the duration was recorded until the hind limbs dropped from the lid. Each experiment was repeated 3 times and the longest duration was recorded. For Longa score, rating scale from 0 to 4 was defined from the complete absence of neurological deficit to lack of conscious response to noxious stimuli. 0 meant normal, 1 meant to be unable to fully extend on the right forepaw, 2 meant to be circling to the right, 3 meant to fall to the right, and 4 meant less motivation to walk and weak consciousness. For mNSS score, rating scale from 0 to 18 was defined from the complete absence of neurological deficit to serious injury. 0 meant normal, 1 to 6 meant mild injury, 7 to 12 meant moderate injury, and 13 to 18 meant severe injury. For modified Bederson's scores, rating scale from 0 to 5 was defined from the complete absence of neurological deficit to lack of conscious response to noxious stimuli. 0 meant normal, 1 meant to be unable to flex the forelimb, 2 meant to be unable to flex the forelimb and decreased resistance to lateral push, 3 meant to be unidirectional circling, 4 meant longitudinal spinning or seizure activity, and 5 meant no movement.

Determination of Infarct Volume

Mice were euthanized after the evaluation of neurobehavioral deficits. The brain was carefully cut into four 2-mm-thick coronal sections from frontal tip using a slicer. The fresh brain slices were immersed in 2% TTC solution at 37°C for 25 minutes and were fixed in 10% formaldehyde. Images were acquired by SZX7 stereoscopic microscope (OLYMPUS, Japan). Contralateral hemispheric areas and ipsilateral noninfarcted areas were measured using Image-Pro Plus 6.0 image analysis software under double-blind conditions. Area was multiplied by the thickness (2 mm) of the brain sections to obtain volume. Infarct volume ratio was calculated using the following formula: infarct volume ratio (%) = (contralateral hemispheric volume − ipsilateral noninfarcted volume)/contralateral hemispheric volume × 100%.[13]

Assessment of Hemorrhagic Transformation

Mice were injected intravenously with 2% Evans blue (0.160 g/kg) to assess hemorrhagic transformation at 22 hours after ischemia. After 2 hours, mice were transcardially perfused with saline and the brain was quickly removed. Hemorrhagic transformation was then assessed by the Heidelberg Bleeding Classification under double-blind conditions.[14] Briefly, hemorrhagic transformation events were scored as: 0 meant no blood; 1 meant small petechiae along the margins of the infarct; 2 meant more confluent petechiae within the infarcted area, but without space occupying effect; 3 meant blood clot not exceeding 30% of infarct area with some mild space-occupying effect; and 4 meant dense blood clots exceeding 30% of infarct area with significant space-occupying effect.

Histopathological Detection through Hematoxylin and Eosin Stain and Nissl Stain

The brain sections of Bregma −4 to −6 mm were fixed by formalin, embedded by paraffin, and coronally sliced into 5-μm sections. For H&E stain, the sections were stained with hematoxylin for 15 minutes and eosin for 5 minutes. After staining, the sections were rinsed with distilled water, dehydrated, and mounted. For Nissl stain, the sections were stained with Nissl stain solution for 5 minutes and rinsed with distilled water, dehydrated, and mounted. Random five regions of cortex and striatum were photographed using VS200 Slide View (OLYMPUS, Japan) microscope. Cell number or Nissl body number per field was counted using ImageJ image analysis software under double-blind conditions.

Immunofluorescent Analysis

Immunofluorescence analysis was conducted to evaluate the expression of GFAP (1:200) and claudin-5 (1:200). Briefly, 5-μm slices were renatured with sodium citrate repair solution, inactivated with 3% hydrogen peroxide, and blocked with host serum (BOSTER, China) for 30 minutes. Then, slices were incubated with the primary antibodies for 30 minutes at room temperature, followed by incubation with Goat anti Rabbit IgG for 10 minutes. After rinsing with phosphate buffer solution, slices were made for immunofluorescence with Novo-light multiple fluorescence immunohistochemical staining kit according to the instruction provided by the manufacturer. VS200 Slide View (OLYMPUS, Japan) microscope-captured image TSA-520 fluorescence for GFAP or claudin-5 with 488-nm excitation and 519-nm emission. Fluorescence intensity per field was counted using ImageJ image analysis software under double-blind conditions.

In Situ Gelatin Zymography

For localization of matrix metallopeptidase 9 (MMP-9) activity, in situ zymography was performed according to previous studies.[15] Briefly, brain tissues were in zinc-buffered fixative for more than 48 hours. Then fixed brains sections of Bregma −4 to −6 mm were cut into 5 μm and deparaffinized in xylene and rehydrated in graded alcohol baths. The sections were incubated with 50 μg/mL of fluorescein-labeled DQ gelatin conjugate, which only produced green fluorescence after digestion with gelatinase at 37°C for 2 hours. After that, the

sections were washed by phosphate buffer solution twice and double-distilled water once and nuclei were stained with DAPI at room temperature for 5 minutes. Random five photomicrographs were taken using an Olympus BX51 microscope plus Olympus DP71 camera (OLYMPUS, Japan). Fluorescence intensity per field was counted using ImageJ image analysis software.

Statistical Analysis

Data are presented as means ± standard error of mean (SEM). After confirming the equal variances, one-way analysis of variance with Tukey's multiple comparisons test analysis was used to compare the difference between groups. Student's *t*-test was used to compare the statistical difference between two groups. $p < 0.05$ was considered statistically significant.

Results

Protection of SalB/Rg1 against Permanent Middle Cerebral Artery Occlusion in a Dose-Dependent Manner

First, protection of SalB/Rg1 was estimated through infarct volume, neurobehavioral deficits and histomorphological examination. As a key indicator for ischemic stroke, infarct volume was detected by TTC stain. In ►**Fig. 1A**, representative staining pictures (left) and quantification data (right) of TTC stain were shown. Compared with Sham group, pMCAO mice with vehicle without SalB/Rg1 treatment (0 mg/kg group) showed a very pronounced infarct volume ($41.22 \pm 3.31\%$, $p < 0.001$). Compared with 0 mg/kg group, both 3.125 mg/kg SalB/Rg1 ($29.10 \pm 2.60\%$, $p < 0.05$) and 6.250 mg/kg SalB/Rg1 ($25.57 \pm 3.14\%$, $p < 0.01$) reduced infarct volume significantly.

Longa score, hanging time and mNSS score were used to evaluate neurobehavioral deficits according to motor, sense, reflex, and balance (►**Fig. 1B**). Compared with Sham group, 0 mg/kg group showed significantly higher Longa score (3 ± 0 vs. 0 ± 0, $p < 0.001$). Longa score of 3.125 mg/kg SalB/Rg1 group (1.75 ± 0.48, $p < 0.05$) or 6.250 mg/kg SalB/Rg1 group (1.57 ± 0.37, $p < 0.05$) was considerably downregulated comparing with 0 mg/kg group. Similar with Longa score, SalB/Rg1 also improved neurobehavioral outcome detected by hanging time and mNSS score.

Because of neurobehavioral improvement of SalB/Rg1 against stroke, the underlying protection of SalB/Rg1 on relative cortex and striatum was further evaluated following. Representative H&E stain of coronal brain section (left) and quantification of cell number (right) were showed in ►**Fig. 2A**. Cortex and striatum in large magnification were

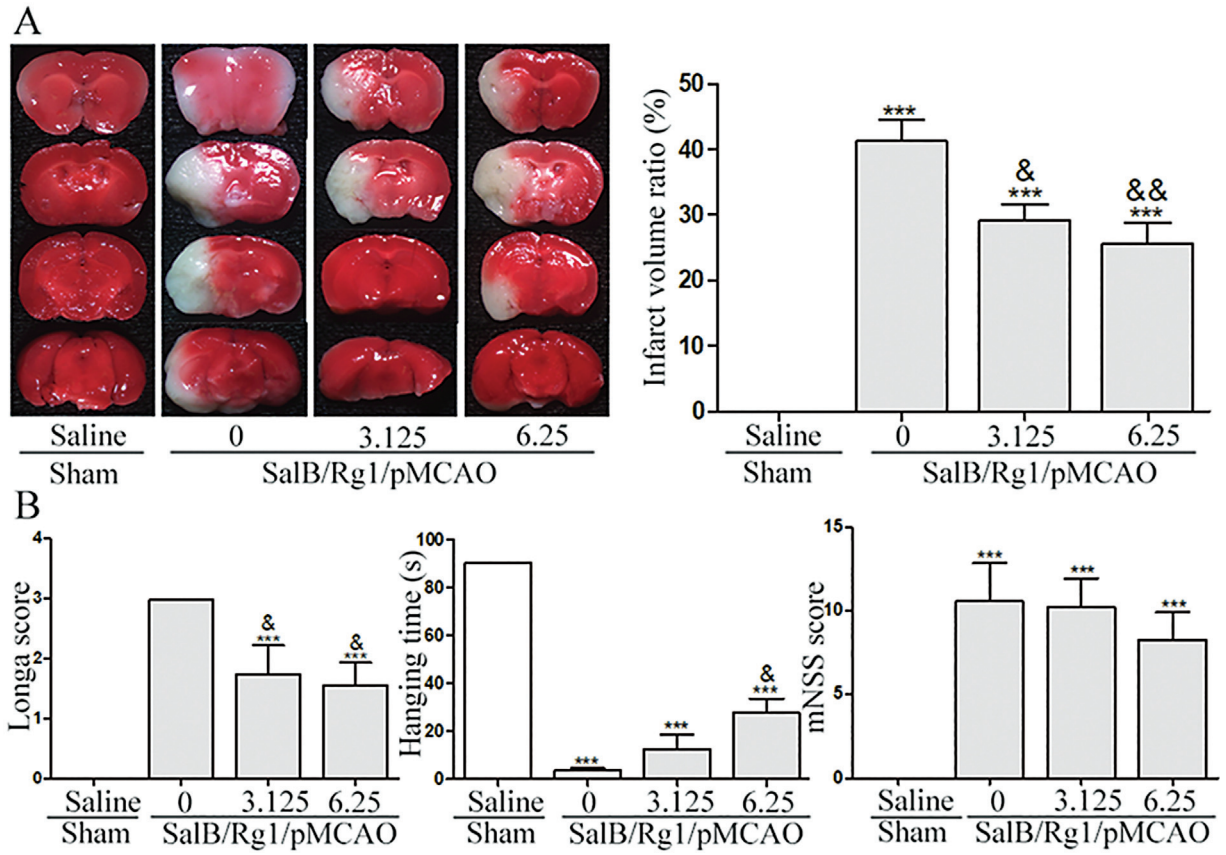

Fig. 1 SalB/Rg1 downregulated the infarct volume and improved neurobehavioral deficits in a dose-dependent manner. (**A**) Representative pictures (left) and quantification (right) of brain with TTC stain. (**B**) Quantification of Longa score, hanging time and mNSS score. Data were expressed with mean ± standard error of mean, $n = 8$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Sham group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus 0 mg/kg group. mNSS, modified neurological severity; pMCAO, permanent middle cerebral artery occlusion; SalB/Rg1, salvianolic acid B and ginsenoside Rg1 combination; TTC, 2,3,5-triphenyltetrazolium chloride.

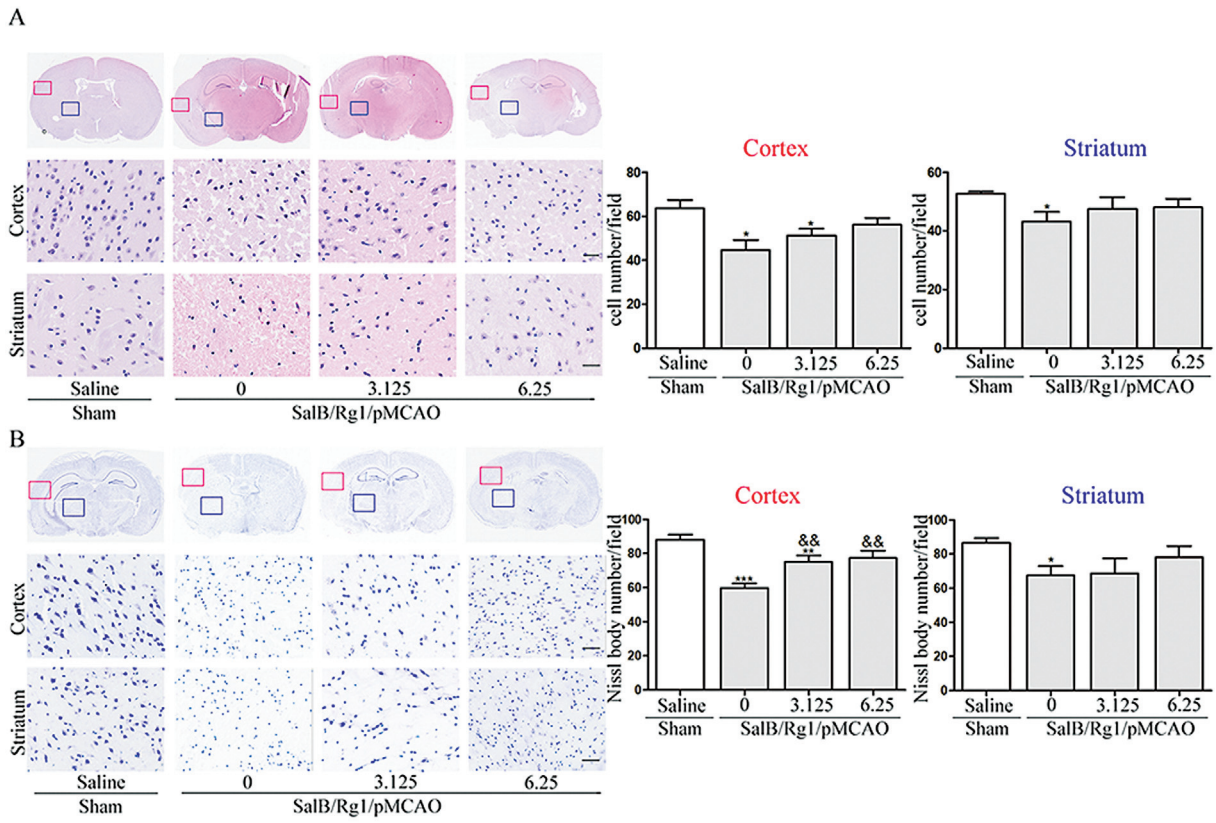

Fig. 2 SalB/Rg1 attenuates cell damage on pMCAO mice in a dose-dependent manner. (**A**) Representative images of whole-brain and subregions including cortex and striatum with H&E stain (left) and quantification of cell numbers per field on cortex and striatum by H&E staining (right). (**B**) Representative images of whole brain and subregions including cortex and striatum with Nissl stain (left), and quantification of the number of Nissl bodies per field on cortex and striatum by Nissl stain (right). Data are shown as the mean ± standard error of mean, $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Sham group; $^{\&\&}p < 0.01$ versus 0 mg/kg group. Scale bar = 50 μm. H&E, hematoxylin and eosin; pMCAO, permanent middle cerebral artery occlusion; SalB/Rg1, salvianolic acid B and ginsenoside Rg1 combination.

shown following a whole-brain section at the indicated rectangular box. Cytoarchitecture with densely distinguishable nucleus and homogeneous cytoplasm was well preserved in Sham group, whereas cytoarchitecture with loosely disarranged nucleus and severely vacuolated cytoplasm was detected in 0 mg/kg group. SalB/Rg1 treatment reversed above lesion on cytoarchitectonic organization in comparison with 0 mg/kg group. Furthermore, Nissl stain was conducted to detect Nissl bodies of surviving neurons (►**Fig. 2B**). Based on quantification of Nissl stain, both 3.125 mg/kg SalB/Rg1 ($p < 0.01$) and 6.250 mg/kg SalB/Rg1 ($P < 0.01$) considerably increased the amount of Nissl body on cortex, whereas only upregulated tendency was observed on striatum either through H&E stain or Nissl stain. In addition, the mortality rate was 50% at 0 mg/kg, 37.5% at 3.125 mg/kg and 12.5% at 6.250 mg/kg (►**Supplementary Fig. 3A**).

SalB/Rg1 Held a Wide Therapeutic Time Window against Permanent Middle Cerebral Artery Occlusion

Time window with therapeutic efficiency is an important characteristic to be elucidated for new medicine. Based on the protection of SalB/Rg1 at 0-hour time point when middle cerebral artery was blocked, we further conducted four batches of experiments and extended time point of SalB/Rg1 treatment to 3-, 6-, 9-, and 15-hour step by step. Infarct volumes between vehicle (Saline) and 6.250 mg/kg SalB/Rg1 treatments were compared on pMCAO mice. Representative TTC stain pictures and quantification of infarct volume were shown in ►**Fig. 3A**. SalB/Rg1 reduced infarct volumes of 26.32 ± 2.53% ($p < 0.05$) at 3 hours, 18.09 ± 2.01% at 6 hours, 25.49 ± 2.03% ($p < 0.05$) at 9 hours, and 18.68 ± 3.06% at 15 hours relative to Saline group. Meanwhile, SalB/Rg1 reduced mortality rate of 22.2% at 6 hours and 11.1% at 15 hours (►**Supplementary Fig. 3B**), respectively. Then, the protection of SalB/Rg1 on cortex and striatum was evaluated through H&E stain (►**Fig. 3B**) and Nissl stain (►**Fig. 3C**). There was significant difference on cell number between Saline and SalB/Rg1 groups on cortex (96.34 ± 4.80 vs. 77.64 ± 4.78, $p < 0.05$) detected by H&E stain at 3 hours. Similar with the above phenomenon at 0 hour, the protection of SalB/Rg1 on cortex is more significant than on striatum.

SalB/Rg1 Alleviated Hemorrhagic Transformation after Ischemic Stroke

Hemorrhagic transformation was a common complication in acute ischemic stroke with large vessel occlusion, which was exacerbated by reperfusion therapy. The effect of SalB/Rg1 was evaluated on hemorrhagic transformation induced by dextrose plus reperfusion. Intravenous injection of Evans blue was used to facilitate the observation of bleeding points, which

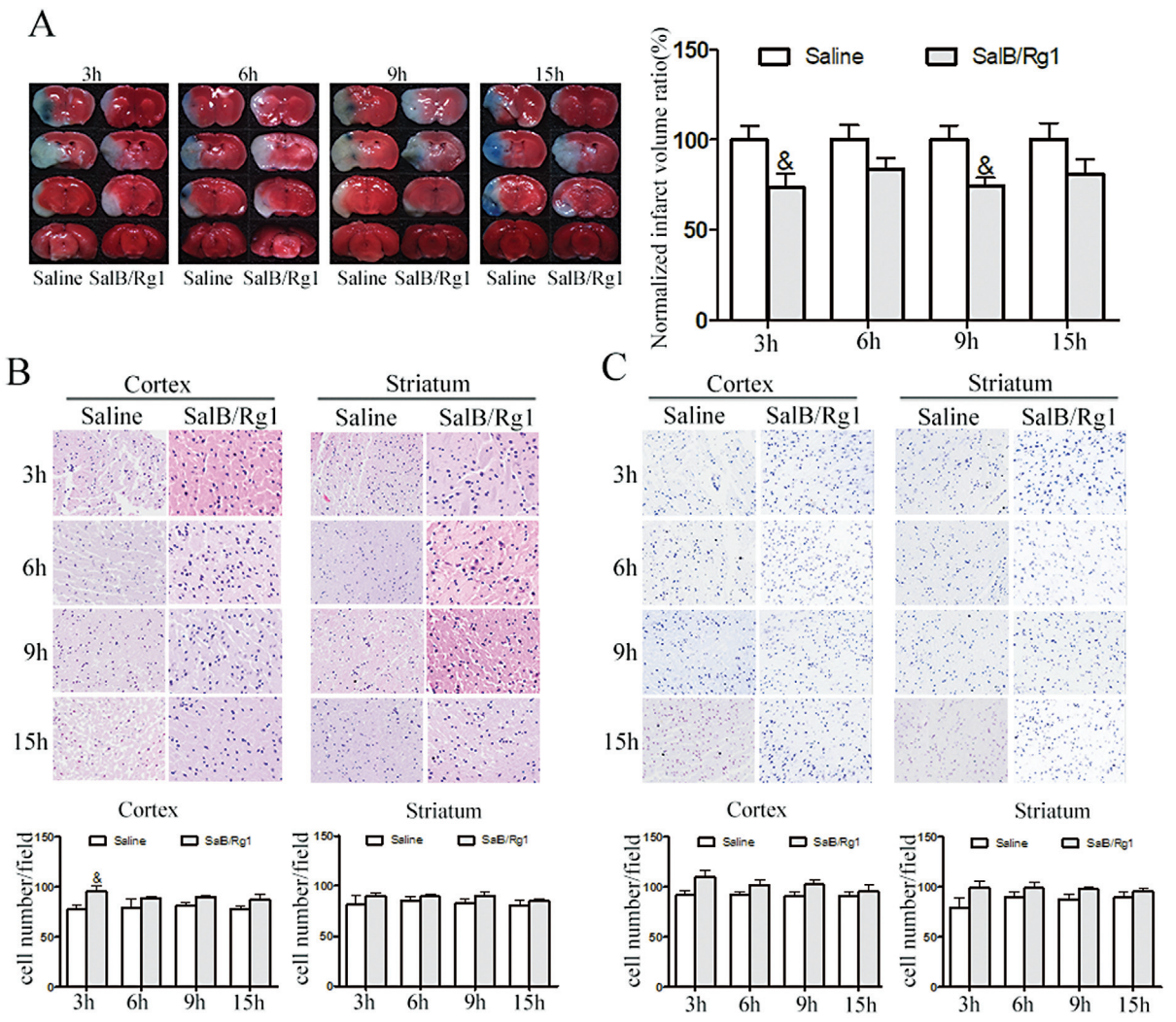

Fig. 3 SalB/Rg1 held a wide therapeutic time window. (**A**) Representative images of TTC stain (left) and quantification of infarct volume (right). (**B**) Representative images of cortex and striatum with H&E stain (up), and quantification of cells numbers per field on cortex and striatum by H&E (down). (**C**) Representative images of cortex and striatum with Nissl stain (up), and quantification of cells numbers per field on cortex and striatum by Nissl stain (down). $n = 9$ for each group. $^{\&}p < 0.05$ versus Saline group. Scale bar = 50 μm. H&E, hematoxylin and eosin; SalB/Rg1, salvianolic acid B and ginsenoside Rg1 combination; TTC, 2,3,5-triphenyltetrazolium chloride.

always located at the edge of Evans blue staining (►**Fig. 4A**). Hemorrhagic scores were identified according to the number and range of bleeding point. Bleeding points in HT group were considerably obvious. Compared with HT group, SalB/Rg1 significantly downregulated hemorrhage score (1.5 ± 0.20 vs. 2.22 ± 0.22, $p < 0.05$). Meanwhile, as shown in the ►**Supplementary Fig. 3C**, SalB/Rg1 reduced the mortality rate of hemorrhagic transformation (40% vs. 50%).

Similar with the protection of SalB/Rg1 on pMCAO mice, SalB/Rg1 also significantly reduced infarct volume ($26.45 \pm 2.21\%$ vs. $34.74 \pm 2.78\%$, $p < 0.05$, ►**Fig. 4B**) and showed improved tendency on neurobehavioral outcome detected by modified Bederson's scores, Longa score, hanging time, and mNSS score (►**Fig. 4C**). Maintenance of the integrity for neurovascular unit plays important role against hemorrhagic transformation during stroke. Because the bleeding point located around the edge around the area with Evans blue staining, the following evaluation on neurovascular unit was conducted in this area.

SalB/Rg1 Maintained Neurovascular Unit with Inhibition on Matrix Metallopeptidase 9 Activity

Astrocytes and endothelial cells are two major cell types of neurovascular unit and play crucial roles in preventing hemorrhagic transformation. In the present study, GFAP was used as the marker to evaluate astrocyte activation, and claudin-5 was used as the marker to evaluate the connection between endothelial cells. Both GFAP (►**Fig. 5A**) and claudin-5 (►**Fig. 5B**) were detected using immunofluorescence analysis. The GFAP fluorescence was significantly enhanced in HT group as compared with Sham group, which fluorescence distributed loosely and uniformly. With SalB/Rg1 treatment, GFAP fluorescence was considerably weakened and quantification further confirmed this downregulation (232.36 ± 14.21 vs. 304.03 ± 61.01, $p < 0.05$) in comparation with HT group. Contrary to the regulation of GFAP, the fluorescence intensity of claudin-5 was significantly increased after SalB/Rg1 treatment comparing with HT group (197.26 ± 26.57 vs. 95.98 ± 18.41 $p < 0.05$). Attenuation of astrocyte activation and enhancement of

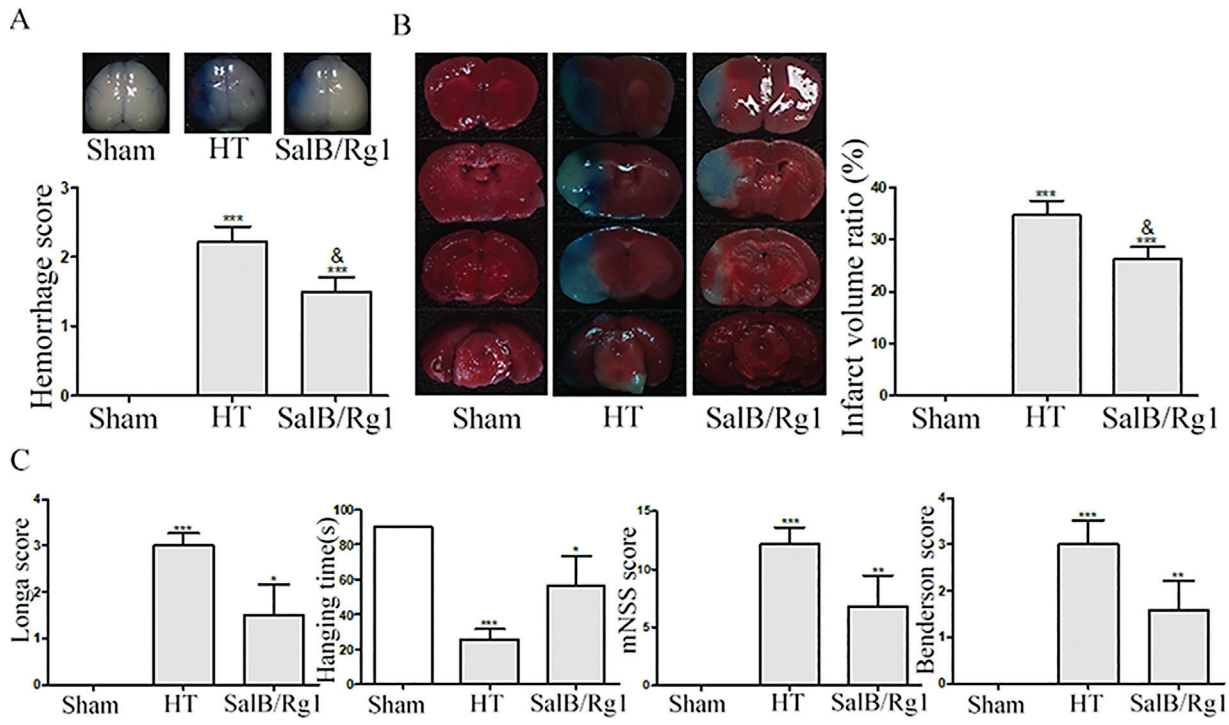

Fig. 4 SalB/Rg1 alleviated hemorrhagic transformation. (**A**) Representative images (up) and quantification data (down) of hemorrhagic score. (**B**) Representative pictures (left) and quantification (right) of brain with TTC stain. (**C**) Quantification of hanging time, Longa score, mNSS score and Bederson's score. Data are shown as the mean ± standard error of mean, $n = 10$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Sham group; $^{\&}p < 0.05$ versus HT group. mNSS, modified neurological severity; SalB/Rg1, salvianolic acid B and ginsenoside Rg1 combination; TTC, 2,3,5-triphenyltetrazolium chloride.

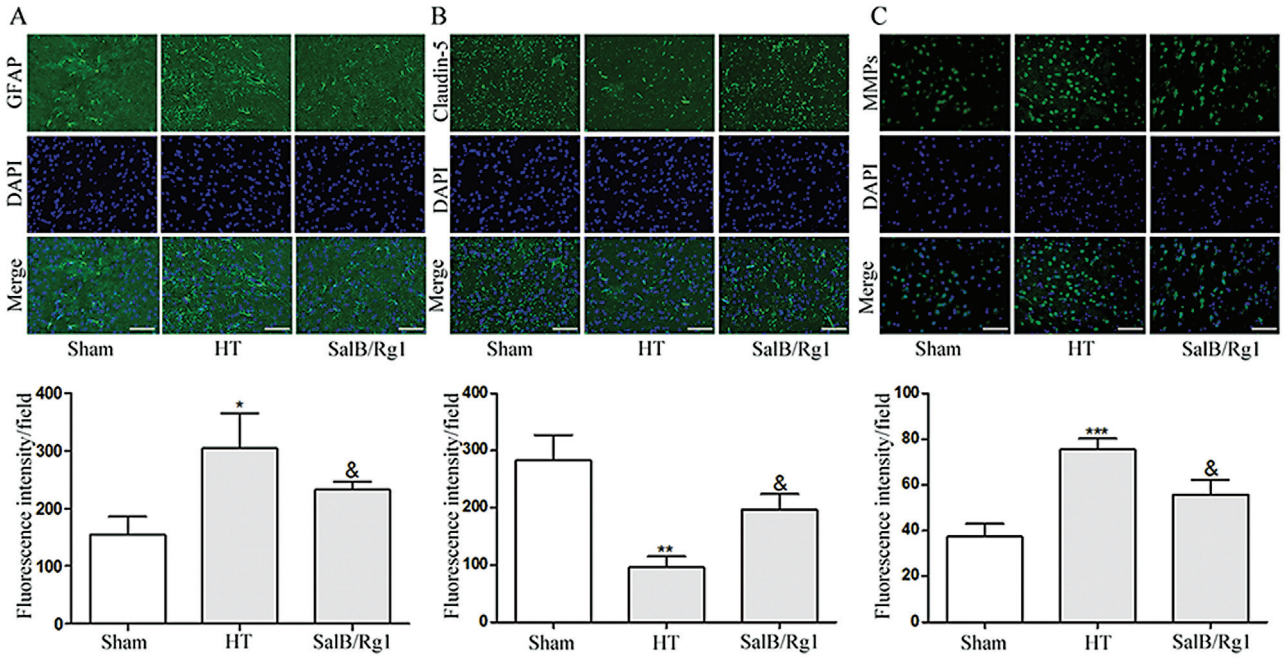

Fig. 5 SalB/Rg1 maintained neurovascular unit with inhibition on MMP-9 activity. (**A**) Representative images (up) and quantification (down) of GFAP (green) and DAPI (blue). (**B**) Representative images (up) and quantification (down) of claudin-5 (green) and DAPI (blue). Scale bar = 50 μm. (**C**) Representative images (up) and quantification (down) of MMPs activity detected by in situ gelatin zymography. Scale bar = 25 μm. Data are shown as the mean ± standard error of mean, $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Sham group; $^{\&}p < 0.05$ versus HT group. GFAP. glial fibrillary acidic protein; MMP-9, matrix metallopeptidase 9; SalB/Rg1, salvianolic acid B and ginsenoside Rg1 combination.

connection between endothelial cells indicated the recovery of the integrity of neurovascular unit.

MMP-9 not only involved activation of astrocyte but also degeneration of connection between endothelial cells. Used in situ zymography, MMP-9 activity was evaluated at the same bleeding area where the detection for GFAP and claudin-5 (►**Fig. 5C**). Consistent with our previous report,[16] SalB/Rg1 also considerably decreased gelatinase activity (green fluorescence) compared with HT group (57.18 ± 7.68 vs. 75.48 ± 4.61, $p < 0.05$).

Discussion

First, the therapeutic effects of SalB/Rg1 on stroke were identified not only on histopathological analysis, but also on neurological outcomes in a dose-dependent manner. Second, time window up to 9 hours for SalB/Rg1 treatment was identified with significant protection. Finally, SalB/Rg1 attenuated hemorrhagic transformation after stroke by maintaining neurovascular unit integrity and inhibiting MMP-9 activity.

Either recanalization through intravenous thrombolysis or through mechanical thrombectomy is time-dependent, which greatly limits the number of patient benefits from above therapeutic strategies.[17] Is it possible to develop new candidate drug acts as a trailbreaker to enlarge time window for recanalization? Now, the relatively promising neuroprotective agent for ischemic stroke is PSD-95 protein inhibitor (NA-1), which halted incorporation of the ischemic penumbra into the necrotic core by targeting excitotoxic mechanisms.[18] The superiority of NA-1 keeping penumbra from conversion into necrotic core expanded the time window for patients to receive mechanical endovascular thrombectomy significantly.[19] SalB and Rg1 are main active ingredients of some commercial preparation products in China. Similar with NA-1, SalB/Rg1 reduced approximately 20% infarct volumes and persevered penumbra until 15 hours after ischemia onset. It is promising to elucidate whether SalB/Rg1 is a new candidate, similar with NA-1, to elongate the time window for intravenous thrombolysis and thrombectomy in the future.

Hemorrhagic transformation is manifested as natural progression of cardiogenic stroke or a complication of recanalization, which causes neurological deterioration subsequently.[20] Strong experimental and clinical evidence linked the risks of bleeding to neurovascular unit dysfunction.[21] The neurovascular unit is a functionally and structurally interdependent multicellular complex composed of neurons, nerve glia cells, endothelial cells, pericytes, and extracellular matrix.[22] During homeostasis, astrocytes extend end-feet processes to cover the surface of cerebral blood vessels with a ratio of approximately 99%, thereby interacting with endothelial cells to maintain the integrity of neurovascular unit.[23] After ischemia, the detachment of activated astrocyte endfeet from vessels and degradation of tight junction proteins between endothelial cells destroy the integrity of neurovascular unit jointly, resulting in hemorrhagic transformation.[24] Among cytoskeleton-building proteins, GFAP is a commonly used marker for astrocyte activation due to ischemic stroke.[25] Claudin-5 is the most abundant tight junction protein between cerebral microvascular endothelial cells and is also used as a potential marker for early hemorrhagic transformation after ischemic stroke.[26]

During ischemia, matrix metalloproteinases cause neurovascular unit dysfunction by disrupting tight junctions between endothelial cells and between endothelial cells and astrocytes.[27] MMP-9 plays an important role in the genesis of hemorrhagic transformation.[28] We previously reported that SalB was a competitive inhibitor of MMP-9.[16] Therefore, the inhibition of MMP activity, downregulation of GFAP expression and maintenance of claudin-5 abundance by SalB/Rg1 protected the integrity of neurovascular unit considerably. Compared with single component, the multicomponent and multitarget served as a novel therapeutic measure to enhance the outcomes of clinical stroke treatment.[29] In future research, it will be demonstrated whether SalB/Rg1 prolong the treatment time window of tPA and reduce hemorrhagic transformation.

Conclusion

Collectively, SalB/Rg1 not only decreased infarct volume with longtime treatment window, but also prohibited the progression of neurological deficits in a dose-dependent manner. Another advantage of SalB/Rg1 against hemorrhagic transformation was related with its protection on neurovascular unit.

Animal Ethics Approval

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica (IACUC No.: 2021-04-GDA-77).

CRediT Authorship Contribution Statement

R.X.: Conceptualization, methodology, data curation, and writing original draft. Y.W.: Conceptualization, methodology, resources, data curation, investigation. L.W.: Conceptualization, methodology. H.S., Y.J. and H.Y.: Resources, data curation, investigation, software. B.J. and R.L.: Funding acquisition, supervision, writing review, and editing.

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Conflict of interest

The authors declare no conflict of interest.

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