ONLINE SUPPLEMENT

Supplemental Methods

Animals

Male C57BL/6 mice were purchased from Charles River (Wilmington, DE, USA). 8- to 10-week-old, 20-25g body weight, age-matched male littermates were used in this study. All mice were randomly assigned to each experiment. All mice were kept in pathogen-free conditions and housed under a 12-h inverted light-dark cycle with access to food and water ad libitum. Reporting of this study complied with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines.[1, 2]

Pertussis toxin administration

Pertussis toxin (List Biological laboratories, Campbell, CA) was dissolved in distilled-deionized water followed by dilution with phosphate buffer saline (PBS) under sterile conditions. And PT was administrated intraperitoneally at a dosage of 250 ng or 500 ng (1ng/μl) per mouse immediately after surgery. The same volume PBS was given in the vehicle group.

ICH induction

ICH in mouse was induced by intra-striatal injection of collagenase as described previously.[3, 4] Mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate (30 mg/kg). Briefly, mice were immobilized onto a stereotactic frame and a 1-mm-diameter hole was drilled on the right side of skull (2.3 mm lateral to midline and 0.5 mm anterior to bregma). 0.0375U Type Ⅳ collagenase (Sigma, St.Louis, MO) in 0.5 μl saline were injected at a depth of 3.7 mm under the skull. Sham mice were subjected to the same surgical procedure with no collagenase injection. Body temperature was maintained at 37.0 ± 0.5°C during the surgery.

Middle cerebral artery occlusion (MCAO) procedure

The model of transient 60-min intraluminal occlusion of MCA was conducted as previously described.[5] The mice were anesthetized with 5% chloral hydrate (30 mg/kg). The left common carotid artery, the external carotid artery and the internal carotid artery were exposed and then isolated and ligated. A standardized silicone rubber-coated nylon monofilament (RWD Life Science, Shenzhen, China) was inserted

into the beginning of the left MCA to occlude circulation for 60 min and then allow reperfusion. Sham mice were subjected to the same surgical procedure, but the filament was not advanced far enough to occlude the MCA. Cerebral blood flow (CBF) was monitored by a laser Doppler probe (model P10, Moor Instruments, Wilmington, DE, USA) for 5 min both before and after MCAO as well as during the first 10 min of reperfusion. Animals that did not show a CBF reduction of at least 75% of baseline level were excluded from further experiments. The body temperature was maintained at 37.0 ± 0.5 °C during surgeries.

Neurological deficit assessment

Neurological tests were performed by investigators blinded to the treatment groups. The modified Neurological Severity Score (mNSS) test consisted of motor, sensory, reflex and balance assessments with the highest possible score being 18. The rota-rod test was performed to evaluate systemic motor function, especially for coordination and balance. The mice were placed on the accelerating rotating rod. The speed was increased from 0 to 40 rpm. Mice were tested 3 times daily with a break of at least 5 min. The latency to fall off the rotating rod was recorded and the results were calculated as the average of three times.[3]

Neuroimaging

Magnetic resonance imaging (MRI) was performed using a 7T small-animal MRI (Bruker, Billerica, MA) equipped with a 72 mm linear transmitter coil and mouse surface receiver coil. The mice were under anesthesia by inhalation of 3.5% isoflurane, and maintained by 1.0–2.0% isoflurane in 70% N2O and 30% O2. The T2-weighted images (T2) were performed to assess the lesion volume of ICH model using the following parameters: TR = 4500 ms, TE = 65.5 ms, FOV = 28 × 28 mm^2, image matrix = 256 × 256, slice thickness = 0.5 mm. Susceptibility weighted imaging (SWI) measurements are sensitive to the presence of paramagnetic substances such as iron compounds. The SWI data were obtained with TR 30 ms and TE 10 ms, flip angle = 25°, FOV = 32 ×32 × 16 mm^3, image matrix = 256 × 256. The lesion was drawn using a T2 sequence, and the hematoma was extracted from the SWI sequence with MRIcorN. The hematoma and T2 were transformed to template space by co-registration in SPM8 and overlaid. T2-weighted images of the brain were acquired with a fat-suppressed rapid acquisition with relaxation enhancement sequence (TR = 4000 ms, TE = 60 ms, FOV = 19.2 × 19.2 mm^2, matrix size = 192 × 192, slice thickness = 0.5 mm) to detect

the infarct size of the ischemic stroke model. The MRI data were analyzed with Image J software (National Institutes of Health, Bethesda, MD).

To detect reactive oxygen species (ROS) generation in the brain, living bioluminescence images were captured using IVIS spectrum (Perkin Elmer, Waltham, MA). Mice were injected intraperitoneally with 200 mg/kg luminol (Invitrogen, Carlabad, CA).[6, 7] Bioluminescence images were captured the chemiluminescent intensities within the brain were defined and measured. Data were collected as photons per second per cm2 by Living Image Software (Perkin Elmer, Waltham, MA).

Cortical cerebral blood flow (CBF) measurements

Cortical CBF was monitored by a laser speckle technique, as previously described.[8] Briefly, images were acquired through the laser speckle contrast imager (PeriCam PSI System, Stockholm, Sweden). Mice treated with PT or vehicle were subjected to measurements of CBF at day 3 after MCAO. CBF changes were expressed as a percentage of baseline.

Immunostaining

The immunostaining was performed as we previously described.[3, 7] Frozen slices were made with 8 μm thickness and then permeabilized and blocked in 5% donkey serum consisting of 0.3% Triton X-100. Thereafter, tissue sections were incubated with primary antibodies against CD31 (Abcam, Cambridge, MA), claudin-5 (Invitrogen, Carlabad, CA) or Matrix Metalloproteinase-9 (MMP-9) (Abcam, Cambridge, MA) at 4°C overnight. After washing with PBS, slices were incubated appropriate fluorochrome conjugated secondary antibodies: donkey anti-rabbit 488 (Invitrogen, Carlabad, CA), donkey anti-mouse 546 (Invitrogen, Carlabad, CA), at room temperature for 1 h. Nuclei were co-stained with DAPI (Abcam, Cambridge, MA). Images were captured by microscope (BioTek, Burlington, VT). For claudin-5/MMP-9 coverage, claudin-5-, MMP-9-, and CD31- positive fluorescent areas were measured by Image J (National Institutes of Health, Bethesda, MD). Claudin-5/MMP-9 coverage was defined as the percentage (%) of claudin-5/MMP-9-positive fluorescent area covering CD31-positive vessel area.

Flow cytometry

Flow cytometry was performed to analyze immune cell infiltration of brain. [6, 7] After the brain has been digested and myelin removed, the cells were stained with fluorochrome conjugated antibodies. All antibodies were purchased from BD Biosciences (San Jose, CA) or BioLegend (San Diego, CA), unless otherwise indicated. The following antibodies were used: CD45 (30-F11), Ly6G (1A8), CD11b (M1/70), CD3 (145- 2C11), CD8 (53-6.72), CD4 (GK1.4), NK1.1 (PK136), CD19 (1D3). Flow cytometric measurements were performed on a FACS AriaⅢ (BD Biosciences, San Jose, CA) and analyzed using Flowjo 7.6 software (Informer Technologies, Walnut, CA).

Cytokine array

Inflammatory cytokines in brain tissues were analyzed by Proteome Profiler Mouse XL Cytokine Array. Brain homogenates were prepared from ICH mice treated with or without PT at day 3 after ICH. After the total protein concentration was adjusted to 1 mg/ml, cytokine levels in these samples were detected using a Mouse XL Cytokine Array Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Evaluation of BBB permeability

Evans Blue (EB) dye (Sigma, St. Louis, MO) was used to assess the BBB permeability of all mice in each group as previously described. [9] Briefly, Evans Blue dye (2% in saline, 4ml/kg) was injected as a tracer at day 3 after ICH or MCAO from internal carotid sinus and then waited for 4 hours before sacrifice. The ipsilateral hemisphere was weighed on a scale and then homogenized into a tube with 2 ml of formamide (Sigma, St. Louis, MO). Follow by incubation in a 60 ℃ water bath for 72h. After centrifugation, supernatants were collected and the optical density (OD) at 600 nm was measured by a microplate reader (Thermo Scientific, Varioskan Flash, USA). The concentration of EB was calculated by the following formula: EB content in brain tissue (μg/ g wet brain) = EB concentration × formamide (ml) / wet weight (g).

Statistical analysis

All results were analyzed by investigators blinded to the treatment groups. And the data are expressed as mean ± SEM. Statistical data analyses were performed using Graphpad Prism 8.0 software (GraghPad, San Diego, CA). Two-tailed unpaired Student t-test was used to determine the significance of differences

between two groups. One-way ANOVA followed by Tukey post hoc test was used for 3 or more groups. Two-way ANOVA accompanied by Bonferroni post hoc test was performed for multiple comparisons. P values < 0.05 were considered statistically significant.

References:

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