SUPPLEMENTAL MATERIAL

Supplemental Methods

SAH Animal Model

Adult male Sprague–Dawley rats (n=202, weight 280-330 g) were housed in a 12/ 12 hours light/dark cycle in 25°C room temperature, humidity control, free access to food and water before surgery. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Loma Linda University. Rats for the whole experiment were administrated in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

The SAH model was performed by the endovascular perforation as following previously reported.[1] Briefly, the rats were anesthetized with 3% isoflurane in 65/35% medical air/oxygen through tracheal intubation by a rodent ventilator (Harvard Apparatus, Holliston, MA, USA). Then A sharpened 4-0 monofilament nylon suture was inserted into the left internal carotid artery from the cut of external carotid artery stump until resistance was felt. The suture was advanced further to perforate the bifurcation of left anterior and middle cerebral artery followed by immediate withdrawal. In sham-operated animals, the same procedures were performed except perforation with suture.

Drug Administration

MitoQ was purchased from MedKoo Biosciences (pre-dissolved in a 1:1 ratio of ethanol-water mixture, yielding a concentration of 200mg/mL, Morrisville, NC) and it was dissolved in 1 mL 0.9% sterile NaCl and administered intraperitoneally (i.p.) 1 h after SAH. ML385 (50 pmol/5μl; AOBIOUS, MA, USA) was diluted in DMSO before intracerebroventricular (i.c.v.) injection and administered 24 h before SAH. SAH+vehicle group and SAH+DMSO group received equal volume of solvents respectively with SAH+MitoQ group and SAH+ML385+MitoQ group. Three different formats of Prohibitin2 small interfering ribonucleic acid (PHB2 siRNA, 500 pmol/5 μL RNase-free water, OriGene Technologies, Rockville, MD, USA), scramble siRNA (Scr siRNA, 500 pmol/5 μL, OriGene Technologies, Rockville, MD, USA) was injected i.c.v. at 48 hours pre-surgery. To enhance the gene silence efficiency, 3 different PHB2 siRNA were mixed, listed as follows: (1) 5'GAGCAAGAAUCCUGGCUAUAUCAAG3'; (2) 5'AUCAUGUGAUGGAUUCUUCUGUATC3'; and (3) 5'AGCAUCAUGUGAUGG AUUCUUCUGT3'.

Intracerebroventricular Administration

Intracerebroventricular drug administration was performed as previous reported.[2, 3] Briefly, post-anesthetized rats were placed in a stereotaxic apparatus under 2% isoflurane anesthesia during the whole surgery. A 10-μL Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV) was delivered into the left ventricle through a burr hole at the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the dural surface. All the chemicals were injected with a rate of 0.5

μL/min. The needle was kept for 5 minutes after infusion and then slowly removed.

Severity of SAH

The severity of SAH was blindly measured using the SAH grading scale after euthanasia as previously described.[1, 3] Briefly, the ventral side of rat brains were photographed immediately after euthanasia and evaluated by an independent observer. The sum of six sub-scores based on six corresponding predetermined areas was calculated as total score (maximum 18). Rats with SAH grading scores ≤7 at 24 hours were excluded from this study.

Assessment of Short-term Neurobehavioral Outcomes

18-point scoring system with a modified Garcia scale and another 4- point scoring system with beam balance test were utilized to blindly evaluate as previous described.[3, 4]

Briefly, the modified Garcia test sore (maximum 18) is composed of six subtests, including spontaneous activity, spontaneous movement of four limbs, forelimbs outstretching, vibrissa touch, body proprioception and climbing capacity. The beam balance sore was evaluated from 0 to 4 according to the ability of animals walking on a narrow wooden beam in 1 min. The mean of three consecutive trials was calculated as the final sore.

Assessment of Long-term Neurobehavioral Outcomes

Foot-fault test and rotarod test were performed in the first three weeks after SAH to evaluate sensorimotor coordination and balance as previously described.[5-8] At 21-25 days after SAH water maze was utilized as previous reported.[8, 9]

In brief, foot fault test was performed on a horizontal grid floor. The foot fault was defined as when rats placed an inaccurate limb and fell it through one of the openings in the grid. The number of foot-faults used for statistics was recorded for 2 minutes. Rotarod (Columbus Instruments, Columbus, OH) consists of a rotating horizontal cylinder (7 cm diameter) which is divided into four lanes (9.5 cm wide). The cylinder stared respectively at 5 revolutions per minute (RPM) and 10 RPM for rats to keep walking forward. The acceleration was set on 2 RPM every 5 seconds. Latency to fall off was recorded by a photobeam circuit. The Morris water maze was performed in a circular metal tank which inside was filled with 37℃ stained black water. A platform was placed at the center of one of the quadrants. In the test, after 4 trials of training with different set of start locations, the platform was submerged requiring rats to find out. An overhead camera and a computerized tracking system (Noldus Ethovision; Noldus, Tacoma, WA, USA) was used to recored the whole swim path, escape latency and swim distance. During the spatial learning memory test, start locations were semi-randomly set, and the time limitation for each rat to find the platform was 60 seconds. After that, each rat was allowed to stay on the platform for 5 seconds. The probe trial was performed on the last day of water maze. Rats were required to search the preexistent area of the platform after removing the actual one.

Western Blot

Left hemispheres of brain samples were collected 24 h after SAH. Western blot was performed as previous described.[3, 10] Briefly, equal amounts of protein samples with 2× loading buffer were loaded onto each lane of SDS-PAGE gel. After electrophoresis, the protein samples were transferred onto a nitrocellulose membrane. Then the membrane was blocked with a blocking solution for 2 hours. After that, the membrane was incubated overnight with primary antibody at 4℃. The primary antibodies with dilution were listed as follows: anti-Keap1, anti-Histone 3 and anti-CoxIV were all purchased from Proteintech Group (Rosemont, IL) with a dilution of 1:1000. Anti-Nrf2, anti-PHB2 and anti-Bcl-xl were all purchased from Abcam (Cambridge, MA) diluted in 1:1000. Anti-PINK1 (1:1000), anti-LC3B (1:5000) and anti-Bax (1:100) were all purchased in Novus (Littleton, CO). Anti-Parkin (1:2000; Santa Cruz Biotechnology, Dallas, TX), anti-ROMO1 (1:200; Aviva Systems Biology, San Diego, CA), anti-Cleaved caspase-3 (1:500; Cell Signaling Technology, Beverly, MA), anti-β-actin (1:5000; Santa Cruz Biotechnology, Dallas, TX, used as loading control for total and cytoplasmic proteins). Appropriate secondary antibodies were all purchased from Santa Cruz Biotechnology (Dallas, TX) with dilution of 1:5000. After incubation with selected secondary antibody for 2 hours in room temperature, the membrane was colored with an ECL regents (Amersham Biosciences UK Ltd, PA, USA). Detected blot bands were quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD).

Histomorphology Measurement

Double immunofluorescence, Dihydroethidium (DHE), Fluoro-Jade C (FJC) and Nissl's staining were performed respectively. A series of 8 μm slices were prepared in Sham, SAH+vehicle, SAH+MitoQ groups.

Double immunofluorescence staining was performed as previous described.[3, 11] The region of interest is the left basal cortex-facing blood clots. Breifly, during euthanasia, rats were intracardially perfused with 60 ml ice-cold phosphate buffer (PBS) and 60 ml of 10% paraformaldehyde through the upper part of the body. The whole brain was then gathered and fixed in 10% paraformaldehyde for 24 hours followed by 30% sucrose for three days. After freezing in -80℃ for 1 hour, a series of 8 μm thick brain slices were cut on a cryostat (LM3050S; Leica Microsystems, Bannockburn, III, Germany). Double fluorescence labeling was processed with 0.3% triton punching for 10 min, 5% donkey serum blocking for 1 hour and the following primary antibody incubating overnight at 4℃. primary antibodies: Anti-Keap1 (1:100; Proteintech Group, Rosemont, IL), anti-Nrf2 (1:100; Abcam, Cambridge, MA), anti-PHB2 (1 :200; Abcam, Cambridge, MA) and anti-NeuN (1:200, Abcam, Cambridge, MA) were used as primary antibodies. Appropriate fluorescence dye–conjugated secondary antibodies (1:500, Jackson Immunoresearch, West Grove, PA) were incubated for 2 h in room temperature before DAPI (Vector Laboratories, Inc., Burlingame, CA) staining and microscope observation (Leica Microsystems, Germany).

The reagent for DHE staining was purchased from Invitrogen (Grand Island, NY), total 25 mg solid was diluted with 1 mL DMSO as the stocking agent, and then take

25.3 μL in 1 mL DMSO as working agent for experiment as previous reported.[12] The region of interest is the left basal cortex-facing blood clots. Briefly, after 1 hour's incubation with working agent, 10 min of PBS washing was performed for 1 time. Then, dry off the slides in 37℃ for 30 min. Finally, washing 3 times with PBS, each time for 10 min. A fluorescence microscope Leica DMI8 (Leica Microsystems, Buffalo Grove, IL) was used for observation, Image J software was used for evaluation. DHE-positive cells were counted in six sections per brain for the mean at ×200 magnification by an independent observer.

Degenerating neurons were evaluated with FJC staining as previous study.[3, 13] The region of interest for short-term is the left basal cortex-facing blood clots, and for long-term is the hippocampus area of the bleeding hemisphere. According to the manufacturer's instructions, a modified FJC ready-to-dilute staining kit (Biosensis, Thebarton, South Australia) was used for preparing slides. The slides were then dried on a slide warmer at 50℃ for 5 min. After that, the dry slides were cleared by brief immersion in xylene for 5 min. The sections of each group were covered with permount and visualized with fluorescence microscope ×200 magnification by an independent observer. The extent of neuronal death for evaluation was calculated by the average number of FJC-positive neurons in six sections per brain slide with Image J software (National Institutes of Health, Bethesda, MD). For long-term study, as the relative fixed position in hippocampus, only three sections in each group were selected for FJC, other counting process was same with short-term study.

Nissl's staining and evaluation of hippocampus injury was performed as previous description.[8] The region of interest is the hippocampus area of the bleeding hemisphere. Briefly, the prepared slides (16 μm) were prepared and stained with 0.5% cresyl violet (Sigma-Aldrich, St. Louis, Mo) for 2 min and washed with distilled water. After dehydrated with xylene, coverslips with permount was placed for microscope ×200 magnification by an independent observer. Neurons in the different regions of each section (3 rats per group, 6 sections per brain) were counted manually by observers blinded to the treatment conditions.

The neuronal loss was estimated according to established methods.[9] Briefly, the mean number of neurons in the different regions of five areas (250×250-μm grids with 16 μm thick in each area) were counted manually with microscope ×200 magnification. Density: 1 = 3,520 neurons/mm^3. The neuronal density loss = neuronal density of non-injured hemisphere - neuronal density of injured hemisphere.

Assessment of Intracellular MDA Levels

Left basal cortical brain samples of each group were harvested and immediately frozen in liquid nitrogen till use. The lipid peroxidation assay kit purchased from Sigma-Aldrich (St. Louis, Mo) was performed to measure the MDA level as previously reported.[14] Briefly, the reaction of MDA was mixed with thiobarbituric acid (TBA) to form a colorimetric (532 nm) product. After incubated at 95℃ for 60 min and cooled on ice bath for 10 min. 200 μl reaction mixture of each group was collected into a 96 well plate for analysis with microplate reader (Bio-Rad, Hercules, CA). The results were expressed as pmol MDA per milligram protein (pmol/mg protein)

Assessment of Nuclear and Mitochondrial Proteins

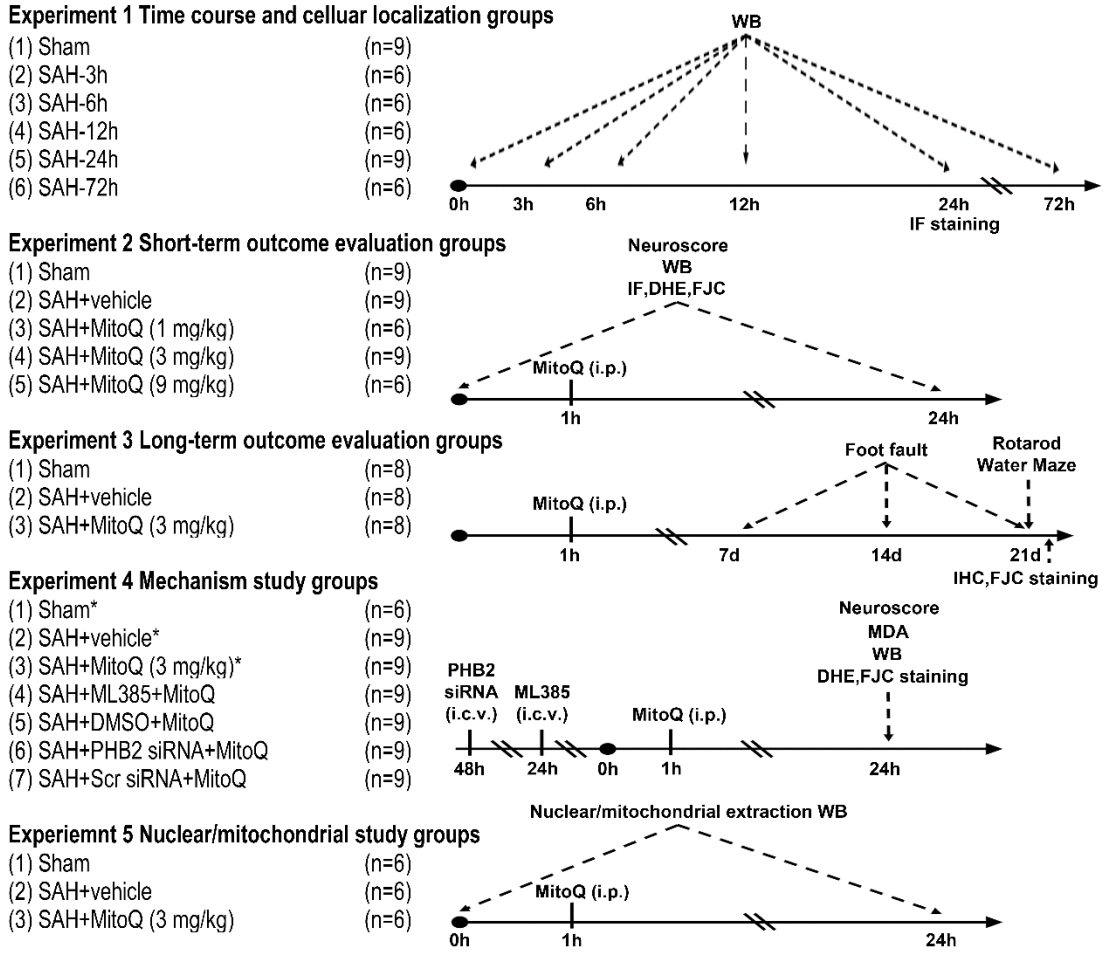
Nuclear and cytoplasmic extraction reagents and mitochondria isolation kit for tissue were both purchased from Thermo Fisher Scientific (Grand Island, NY, USA). The method for two types of extractions was described previously.[15] Left basal cortical brain samples were equally divided into two parts for isolation of nucleus and mitochondria respectively following the instruction manual. Western blot was then performed to detect the proteins expression of each organelle components.

Supplemental References

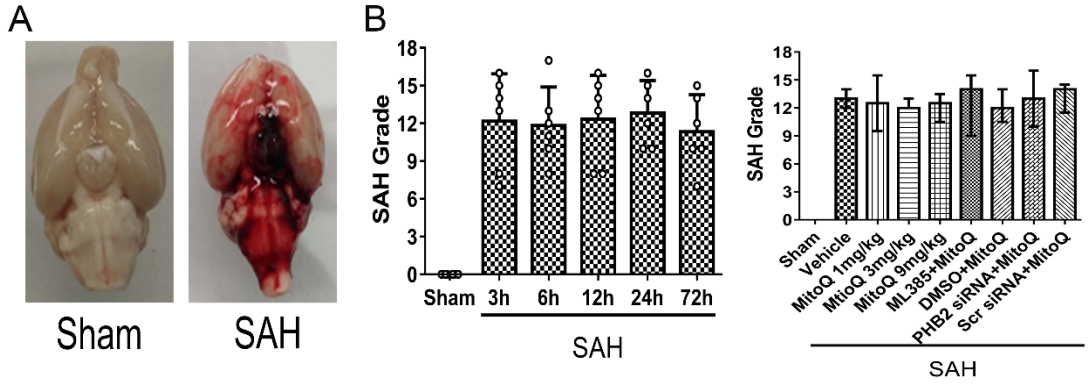
1. Sugawara T, Ayer R, Jadhav V, Zhang JH. A new grading system evaluating bleeding scale in filament perforation subarachnoid hemorrhage rat model. *J. Neurosci. Methods*. 2008;167:327-334
2. He Y, Xu L, Li B, Guo ZN, Hu Q, Guo Z, et al. Macrophage-inducible c-type lectin/spleen tyrosine kinase signaling pathway contributes to neuroinflammation after subarachnoid hemorrhage in rats. *Stroke*. 2015;46:2277-2286
3. Xie Z, Enkhjargal B, Wu L, Zhou K, Sun C, Hu X, et al. Exendin-4 attenuates neuronal death via glp-1r/pi3k/akt pathway in early brain injury after subarachnoid hemorrhage in rats. *Neuropharmacology*. 2017;128:142-151
4. Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke*. 1995;26:627-634; discussion 635
5. Zhou Y, Lekic T, Fathali N, Ostrowski RP, Martin RD, Tang J, et al. Isoflurane posttreatment reduces neonatal hypoxic-ischemic brain injury in rats by the sphingosine-1-phosphate/phosphatidylinositol-3-kinase/akt pathway. *Stroke*. 2010;41:1521-1527
6. Lekic T, Hartman R, Rojas H, Manaenko A, Chen W, Ayer R, et al. Protective effect of melatonin upon neuropathology, striatal function, and memory ability after intracerebral hemorrhage in rats. *J. Neurotrauma*. 2010;27:627-637
7. Hu Q, Vakhmjanin A, Li B, Tang J, Zhang JH. Hyperbaric oxygen therapy fails to reduce hydrocephalus formation following subarachnoid hemorrhage in rats. *Med. Gas Res.* 2014;4:12
8. Sherchan P, Lekic T, Suzuki H, Hasegawa Y, Rolland W, Duris K, et al. Minocycline improves functional outcomes, memory deficits, and histopathology after endovascular perforation-induced subarachnoid hemorrhage in rats. *J. Neurotrauma*. 2011;28:2503-2512
9. Vorhees CV, Williams MT. Morris water maze: Procedures for assessing spatial and related forms of learning and memory. *Nat. Protoc.* 2006;1:848-858
10. Suzuki H, Hasegawa Y, Chen W, Kanamaru K, Zhang JH. Recombinant osteopontin in cerebral vasospasm after subarachnoid hemorrhage. *Ann. Neurol.* 2010;68:650-660

11. Li L, Tao Y, Tang J, Chen Q, Yang Y, Feng Z, et al. A cannabinoid receptor 2 agonist prevents thrombin-induced blood-brain barrier damage via the inhibition of microglial activation and matrix metalloproteinase expression in rats. *Transl Stroke Res*. 2015;6:467-477
12. Yamamoto E, Tamamaki N, Nakamura T, Kataoka K, Tokutomi Y, Dong YF, et al. Excess salt causes cerebral neuronal apoptosis and inflammation in stroke-prone hypertensive rats through angiotensin ii-induced nadph oxidase activation. *Stroke*. 2008;39:3049-3056
13. Xie Z, Huang L, Enkhjargal B, Reis C, Wan W, Tang J, et al. Intranasal administration of recombinant netrin-1 attenuates neuronal apoptosis by activating dcc/appl-1/akt signaling pathway after subarachnoid hemorrhage in rats. *Neuropharmacology*. 2017;119:123-133
14. Yuksel Y, Guven M, Kaymaz B, Sehitoglu MH, Aras AB, Akman T, et al. Effects of aloe vera on spinal cord ischemia-reperfusion injury of rats. *J. Invest. Surg.* 2016;29:389-398
15. Qiu H, Lizano P, Laure L, Sui X, Rashed E, Park JY, et al. H11 kinase/heat shock protein 22 deletion impairs both nuclear and mitochondrial functions of stat3 and accelerates the transition into heart failure on cardiac overload. *Circulation*. 2011;124:406-415

Supplemental Figures and Figure legends

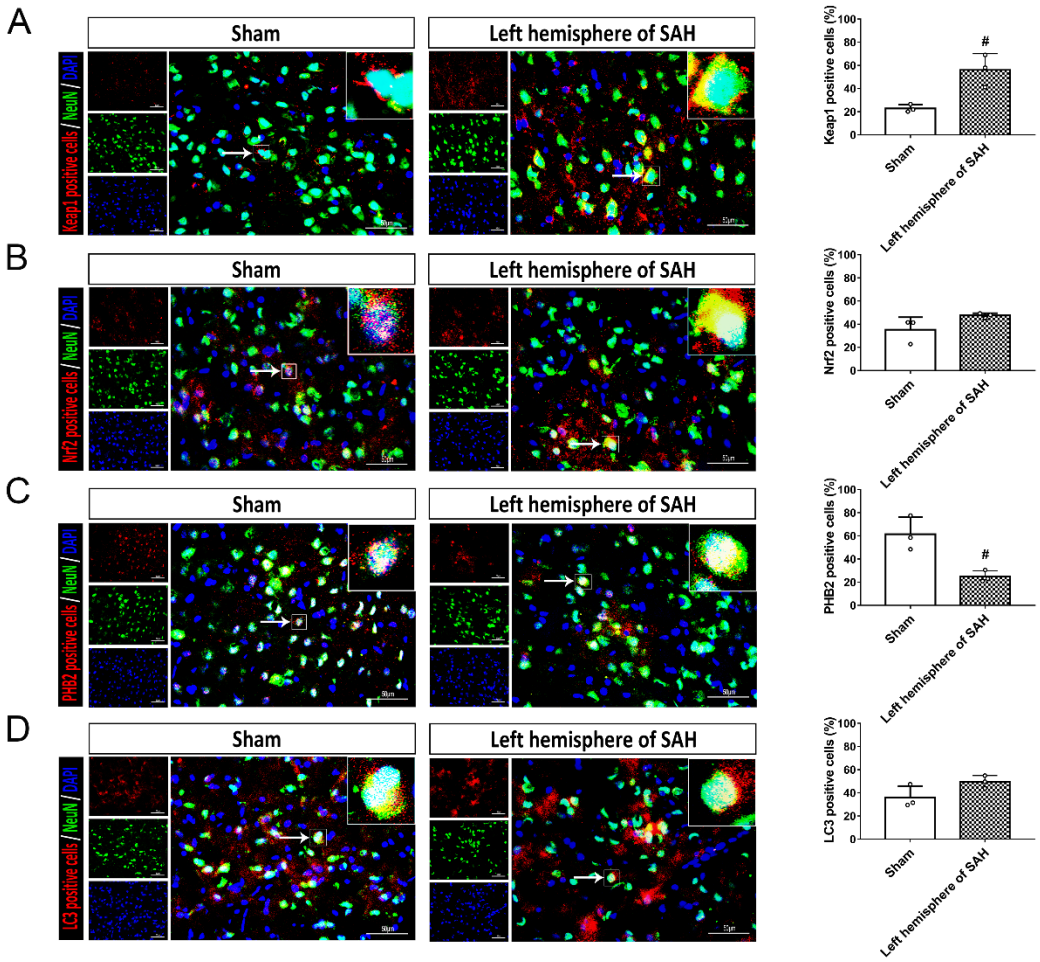


Supplemental Figure I. Experimental protocols and animal groups. * means the shared groups with Experiment 2. Vehicle, 0.9% sterile NaCl; ML385, an inhibitor of Nrf2; DMSO, dimethyl sulfoxide; siRNA, small interfering ribonucleic acid; Scr siRNA, scramble siRNA.

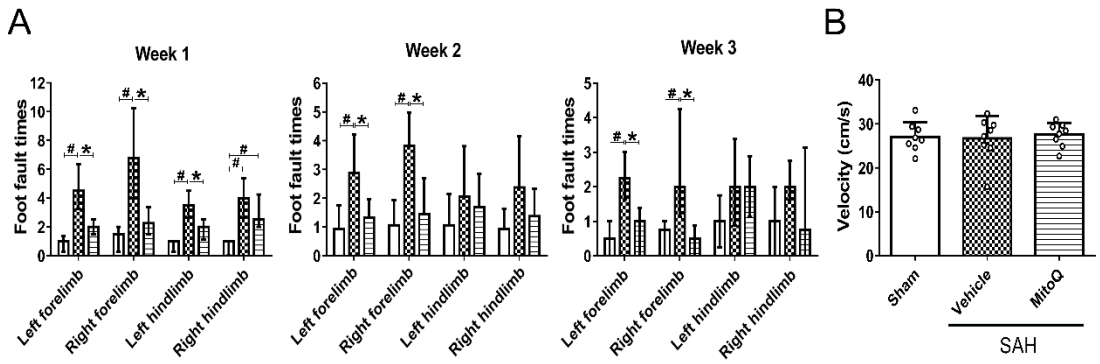


Supplemental Figure II. Subarachnoid hemorrhage (SAH) model and grade. (A) Representative images of Sham and SAH in rats. (B) SAH grade scores of rats in all SAH groups. Vehicle, 0.9% sterile NaCl; ML385, an inhibitor of Nrf2; DMSO,

dimethyl sulfoxide; siRNA, small interfering ribonucleic acid; Scr siRNA, scramble siRNA. Data of time course SAH grade was expressed as the means ± SD using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The other data was expressed as the medians with interquartile range using Kruskal–Wallis test followed by the Dunn's post hoc test.



Supplemental Figure III. Expression of targeted proteins in rat brain. Representative images and quantitative analyses of (A) Keap1, (B) Nrf2, (C) PHB2, (D) LC3 colocalized with neurons in Sham and Left hemisphere of SAH groups. Fluorescence microscope ×400 magnification. Positive cells were counted in six sections per brain slide, n=3 per group. Data were expressed as the means ± SD using Student t test. #P< 0.05 Sham group.



Supplemental Figure IV. Mitoquinone (MitoQ) attenuated long-term neurological deficits after subarachnoid hemorrhage (SAH). (A) Foot fault test of every three weeks. (B) Water maze test related velocity in three groups above. n=8 per group. Data of foot fault test in week1 and week3 were expressed as the medians with interquartile range using Kruskal–Wallis test followed by the Dunn's post hoc test. Other data were expressed as the means ± SD using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. #$P < 0.05$ vs. Sham group; *$P < 0.05$ vs. SAH+vehicle group.

*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	Yes

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	No

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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