SUPPLEMENTAL MATERIAL

Supplemental Methods

Mouse model of intracranial aneurysms

Experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee. We used C57BL/6J and $Kit^{W\text{-}sh/W\text{-}sh}$ transgenic male mice (Jackson Laboratory, Bar Harbor, Maine). Intracranial aneurysms were induced by combining induced systemic hypertension and a single injection of elastase (35.0 milli-units; mU, unless indicated otherwise) into the cerebrospinal fluid at the right basal cistern as we previously described.[24]
This dose of elastase was chosen so that we can achieve 60-90% of the incidence of aneurysm formation, based on our previous studies.[3, 21, 23-25, 31, 32, 52] We used the same lot of elastase for injections of all study groups. We performed a series of test injections for every 10 -15 mice to ensure the correct needle placement as previously described.[50] To induce systemic hypertension, we used deoxycorticosterone acetate (DOCA)-salt hypotension as previously described.[3, 53] DOCA-salt hypertension is a classical method for inducing hypertension that has been successfully used in various species.[3, 53] DOCA-salt hypertension requires left nephrectomy followed by implantation of DOCA pellet one week later; 1% sodium chloride drinking water was started on the same day as the DOCA pellet implantation as previously described.[3, 53] Angiotensin II was not used, as it is not part of the DOCA-salt hypertension.

Evaluation of aneurysm formation and rupture

To detect aneurysmal rupture, two blinded observers performed neurological examinations daily as previously described.[24] Neurological symptoms were scored as follows: 0: normal function; 1: reduced eating or drinking activity demonstrated by a weight loss >2 g of body weight (≈10% weight loss) >24 hours; 2: flexion of the torso and forelimbs on lifting the whole animal by the tail; 3: circling to 1 side with a normal posture at rest; 4: leaning to 1 side at rest; and 5: no spontaneous activity. Mice were euthanized when they developed neurological symptoms (score, 1-5). Aneurysms are defined as a localized outward bulging of the vascular wall (>150% of the control artery).[24] When mice develop neurological symptoms associated with aneurysmal rupture (neurological score: 1-5), we euthanize them immediately (within 4 hours). In both euthanized and dead mice, we inspect the brain samples and verify the presence of aneurysm and hematoma from subarachnoid hemorrhage by examining the Circle of Willis and its major branches under a dissecting microscope (10X).[3] Our study confirmed the specificity and sensitivity of this approach in detecting aneurysmal rupture.[3] Because our previous studies using this model showed that aneurysmal rupture occurs within 3 weeks of aneurysm induction, asymptomatic mice were euthanized 21 days after aneurysm induction as previously described.[23, 25, 31, 32, 51] The brain samples were perfused with phosphate-buffered saline, followed by a gelatin-containing 1% of bromophenol blue dye to visualize cerebral arteries. Two blinded observers assessed the formation of intracranial aneurysms by examining the Circle of Willis and its major branches under a dissecting microscope (10X).

Dosing of drugs

The doses of cromolyn (12.5 or 25 mg/kg/day, i.p.) and C48/80 (2 or 4 mg/kg/day, i.p.) were chosen according to previous published studies in mice.[27, 28] Cromolyn is a clinical used drug against diseases such as asthma and systemic mastocytosis. The dose used in the current study is below the dose used in humans according to surface area conversion.[29]

Tissue collection and immunohistochemistry

To confirm the presence of mast cells in intracranial aneurysms in this model, we stained representative aneurysms for mast cells as previously described.[7, 16] We collected aneurysms from mice at seven days after aneurysm induction, a time point before aneurysmal rupture begins to occur.[3] Mice were sacrificed and perfused with ice-cold saline followed by perfusion with a gelatin-containing 1% of bromophenol blue dye to visualize cerebral arteries. After taking macroscopic pictures of the major cerebral arteries, the brain tissues were fixed with 4% paraformaldehyde for 24 hours and then immersed in 15% sucrose for 24 hours and 30% sucrose for another 24 hours. Then, the tissues were frozen in optimal cutting temperature compound (OCT, Tissue-Tek) at -80˚C. Serial cross-sections (10 μm thick) were mounted on microscope slides (Fisher Scientific Co.). Sections were immunohistochemically stained with monoclonal antibodies to mast cells using anti-mast cells tryptase clone AA1 (DakoCytomation, Carpentaria, CA). The primary antibody was omitted for negative control. Sections were fixed with cold acetone for 20 minutes, and endogenous peroxidases were quenched by incubating sections in 0.3% hydrogen peroxide in methanol. Sections were subsequently blocked with 10% normal serum from host species of the secondary antibody. Sections were incubated in primary antibody overnight at 4°C, followed by incubation with corresponding biotinylated secondary antibodies (Vector Laboratories) and with a complex of avidin-biotin-horseradish peroxidase (Vector Laboratories). Immunoreactivity was visualized by incubating the sections with 0.05% 3,3'-diaminobenzidine (DAB, Vector Laboratories). Nuclei were visualized by counterstaining with aqueous hematoxylin.

Real-time PCR detection of cytokines and enzymes

We collected total RNA samples from cerebral arteries (Circle of Willis, including aneurysms) 5 days after aneurysm induction as previously described.[30, 31] We measured mRNA expression levels of inflammation-related cytokines (AT1R [angiotensin II type I receptor], IL-6 [interleukin-6], MMP-9 [Matrix metallopeptidase 9], and TNF-α [tumor necrosis factor-α]), cathepsin G, chymase, and tryptase. The following primers was used: AT1R, forward 5'- GTT CCT GCT CAC GTG TCT CA -3', reverse 5'- CAT CAG CCA GAT GAT GAT GC -3'; IL-6, forward 5'-CCG GAG AGG AGA CTT CAC AG-3', reverse 5'-GGA AAT TGG GGT AGG AAG GA-3'; MMP-9, forward 5'-AGA CCT GAA AAC CTC CAA CCT CAC-3', reverse 5'-TGT TAT GAT GGT CCC ACT TGA GGC-3'; TNF-α, forward 5'-CCA GAC CCT CAC ACT CAG ATC-3', reverse 5'-CAC TTG GTG GTT TGC TAC GAC-3'; cathepsin G, forward 5'-GCC AGA GCA GGG GAA CAA ATG -3', reverse 5'- GCC TGA GAC AGG GAG TTA GCA G -3'; chymase, forward 5'- ACC ACT GAG AGA GGG TTC ACA GC -3', reverse 5'- GAA GAC TCT GAT GCA CGC AGG TC -3'; tryptase, forward 5'- GCT CCT CTC TTT GAA CCG GAT C -3', reverse 5'- GGT GGG AGA GGC TCG TCA TTA -3' (Integrated DNA Technologies, Coralville, IA). RNA was extracted using the RNeasy Mini Kit (Qiagen, CA) and transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen). The mRNA expression levels were determined using SYBR Green technology (Applied Biosystems, CA).

Quantitative values were obtained from the threshold cycle value (Ct), and the data were analyzed by the $2^{-\Delta\Delta}$CT method. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression was quantified and used as an internal RNA control.

Statistical analysis

Fisher's exact test was used to analyze the incidences of aneurysm formation and rupture rates among groups. 95% confidence interval was calculated for all percentage of incidence rate and rupture rate. Mann-Whitney test was used for the analysis of RT-PCR data. P-values < 0.05 were considered statistically significant. Data are expressed as means ± SD.

Supplemental Figures and Figure Legends

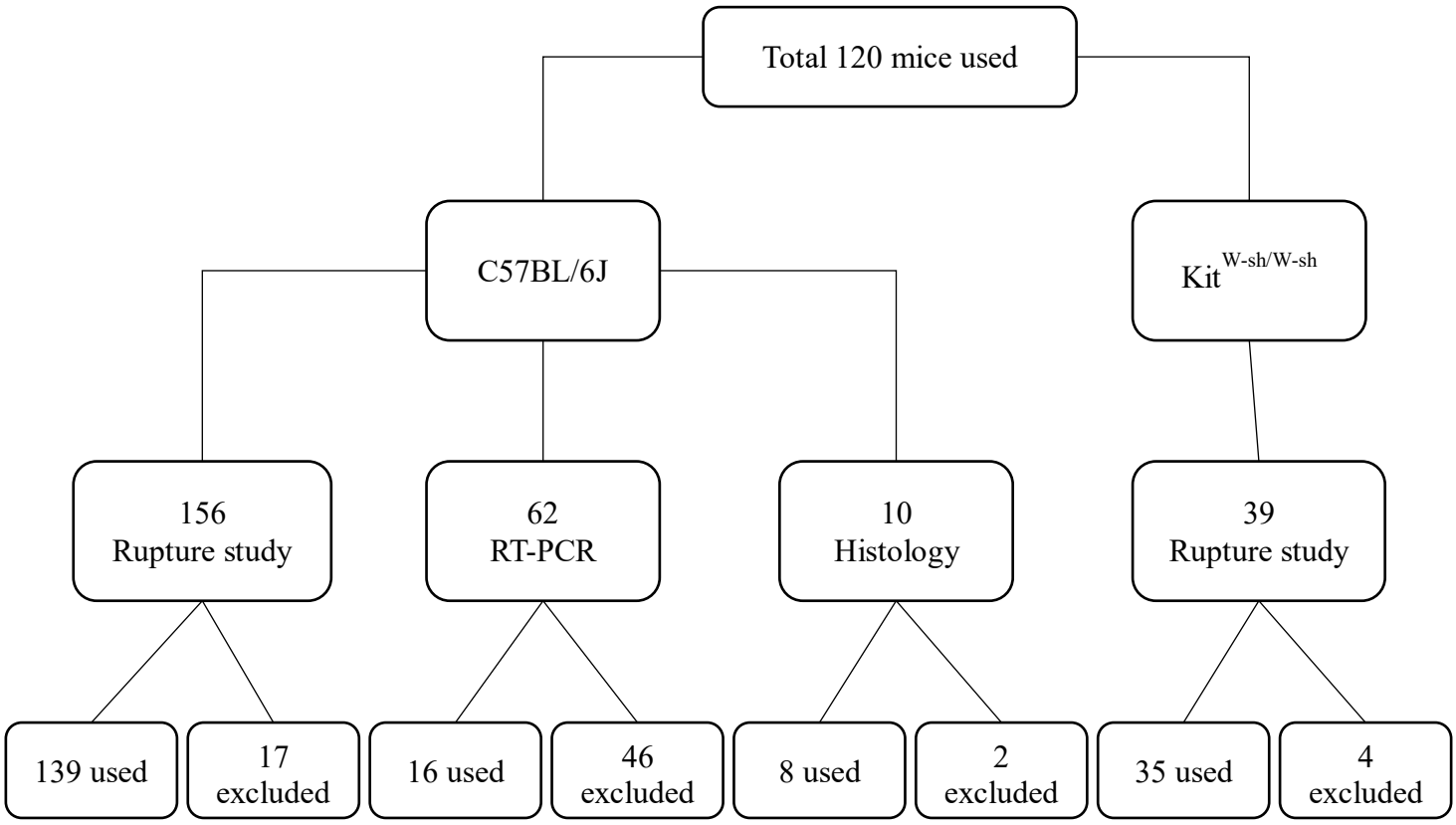

Figure I. **Flow chart of mice used in current study.** The excluded mice in the rupture studies were due to humane sacrifice within a week after the surgery. For PCR studies, we only use tissue of Circle of Willis from mice with or without un-ruptured aneurysms. We excluded mice died early due to rupture of aneurysm (25 mice), and mice with asymptomatic hemorrhage (21 mice).

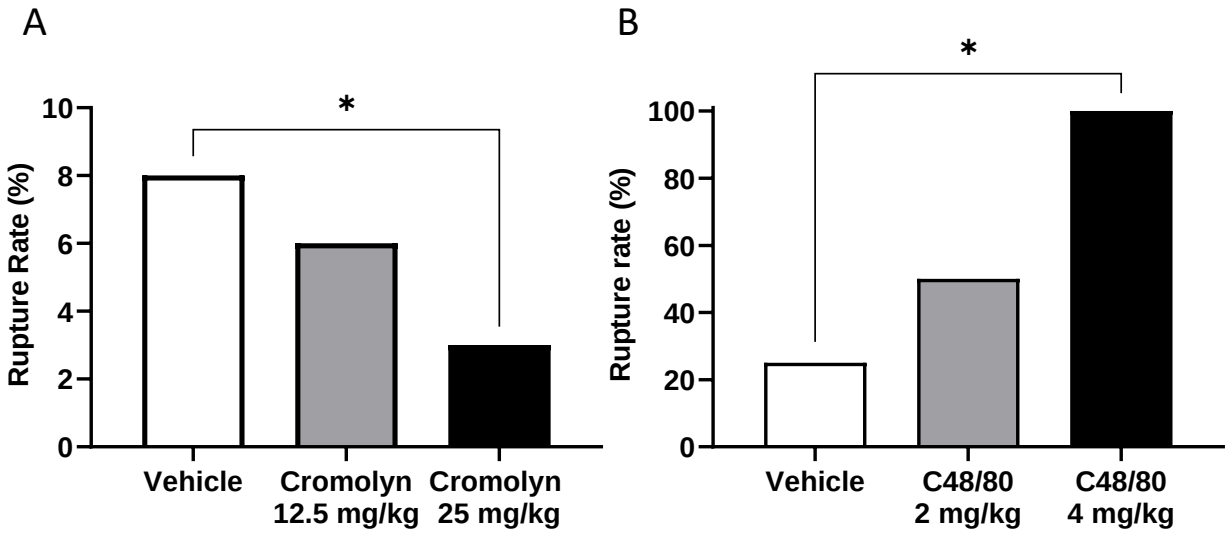

Figure II. A. Pharmacological stabilization of mast cells by cromolyn after aneurysm formation prevented aneurysmal rupture. Aneurysm rupture rate was significantly decreased in full dose cromolyn-treated (25 mg/kg/day) as compared to vehicle-treated mice. * $P < 0.05$. Half-dose cromolyn treatment also reduced rupture rate, which did not reach to the same level as full-dose treatment, suggesting dose-dependent effect of cromolyn. **B. Pharmacological activation of mast cells after aneurysm formation promoted aneurysmal rupture without affecting the aneurysmal formation.** Aneurysm rupture rate was significantly increased in full-dose C48/80-treated (4 mg/kg/day) as compared to vehicle-treated mice. * $P < 0.05$. Half-dose C48/80 treatment also increased rupture rate, which did not reach to the same level as full-dose treatment, suggesting dose-dependent effect of C48/80.

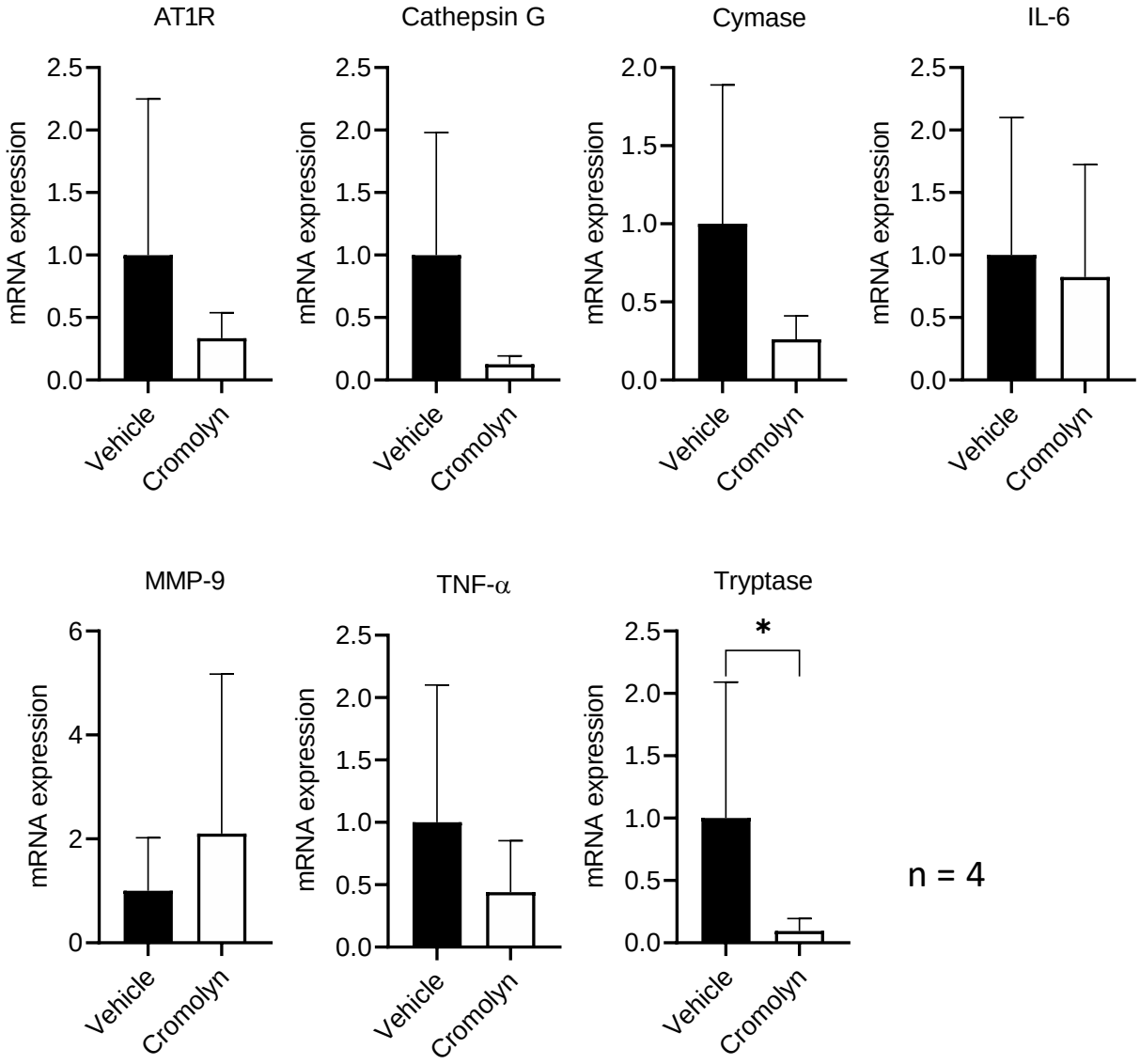

Figure III. Real-Time PCR quantification of mRNA. Cromolyn treatment (25 mg/kg/day) significantly decreased the mRNA expression of tryptase as compared to vehicle-treated mice (Data is expressed as mean ± SD, Mann-Whitney test, * $P < 0.05$, n = 4 for all groups).

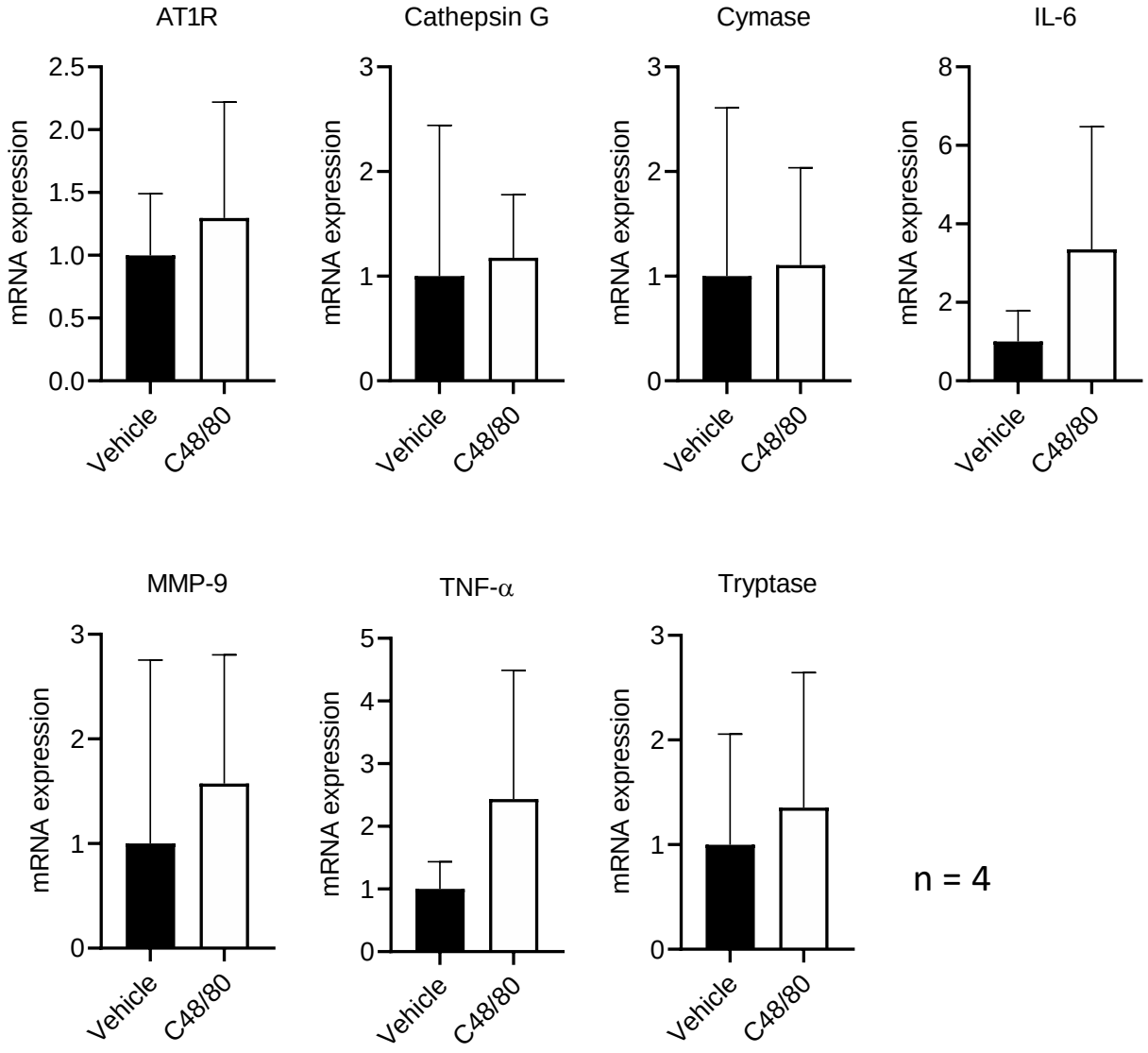

Figure IV. Real-Time PCR quantification of mRNA. C48/80 treatment (4 mg/kg/day) did not change the mRNA expression of tested molecules as compared to vehicle-treated mice (Data is expressed as mean ± SD, Mann-Whitney test, n = 4 for all groups).

*** 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:	Yes

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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