Supplementary Information

Mature adult mice with exercise-preconditioning show better recovery after intracerebral hemorrhage

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Content:

Expanded Materials and Methods:

Animals - In this study, we used 25-week old male C57BL/6 J mice (Jackson Laboratory, USA). Mice were housed in a specific pathogen-free conditioned 12-h light/dark cycle room (light on between 8:00 AM and 8:00 PM) with free access to food and water throughout the experiment. All experimental procedures were reviewed and approved by a Subcommittee for Research Animal Care of the Massachusetts General Hospital IACUC (Institution Animal Care and Use Committee), and we used an institutionally approved animal protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

Treadmill training protocol - The exercise group ran on a treadmill device (Exter 3/6 Treadmill, Columbus Instruments, USA) for 6 weeks (in the afternoon, Monday through Friday). Running speed began with a speed of 2 m/min, with an increase of 2 m/min every 2 minutes, until a maximum speed of 10 m/min was reached. The duration of the total exercise was initially set at 20 minutes and increased daily by 10 minutes up to 60 minutes for familiarization (see Figure 1a). The sedentary group was placed on the device without running for the same amount of time as the training group. Mice were always placed in the same lane throughout the experiment. To assess adverse events induced by exercise, neurological score was evaluated before and after exercise for the training group. Mice unable to achieve a normal score were to be excluded from the study, but none of the mice in this study met the criteria for disqualification.

ICH induction - After initiating anesthesia using 3.5% isoflurane and maintaining anesthesia using 1.5% isoflurane in 70% N_2O and 30% O_2, mice were placed in a stereotaxic frame with a maintained temperature between 36.5°C and 37.5°C. A 30-gauge needle was inserted into the right striatum through a burr hole in the skull (stereotaxic coordinates; 2.3 mm lateral to the midline, 0.2 mm anterior to the bregma and 3.5 mm depth below the skull). Then, ICH was induced by injection of 0.025 U collagenase type VII (Sigma, St Louis, MO, USA) in 0.5 μL physiological saline, at a rate of 0.2 μL/min. After surgical operation, mice were returned to their home cages and maintained under the same conditions' pre-operation. No animals died due to the ICH operation in this study.

Behavioral tests - Motor and sensorimotor functions of mice were evaluated by the beam-walking test and the adhesive removal test, respectively, by an investigator who was blinded to the grouping. Mice were placed in the testing room at least 30 minutes before starting the tests. In the beam-walking test,

mice were trained three times in the day before surgery for ICH induction. A beam with a 0.5-inch width, 1.1-m length, and 0.5-m height was used. Hindlimb fault rate and walking distance were obtained as the average value from three trials. The performance score of mice was based on an eight-point scale. In the adhesive removal test, at first, mice were placed in the testing box for 1 min to allow them to habituate to the new environment. After that, mice were gently removed from the testing box, and two adhesive tape strips were applied with equal pressure to each paw. The mice were then placed in the testing box again, and their behaviors were observed for 120 seconds. In the corner turning test, mice were placed in the testing room in the early afternoon, at least 30 min before starting the test. Mice were led to a 30° angle corner and were required to turn either to left or right when their whiskers contacted the wall. A total of 10 trials was conducted, and a score was calculated as the number of left turns/all trials × 100. These behavioral tests were always conducted in the early afternoon.

Estimation of lesion volume - The lesion area/volume were estimated by Nissl staining with Cresyl Fast Violet Acetate Certified, DcW-5 (Electron Microscopy Sciences, Hatfield, PA) of coronal brain sections of 20 μm thickness obtained every 200 μm. The lesion area in these sections was measured by quantifying the Nissl staining-negative area by using ImageJ software. Lesion volume was determined by integration of the lesion area in each section over section depth.

Estimation of hematoma volume - The hematoma area/volume were estimated by observation of coronal brain sections of 20 μm thickness obtained every 200 μm. The hematoma area was measured by using ImageJ software. Hematoma volume was determined by integration of the lesion area in each section over section depth.

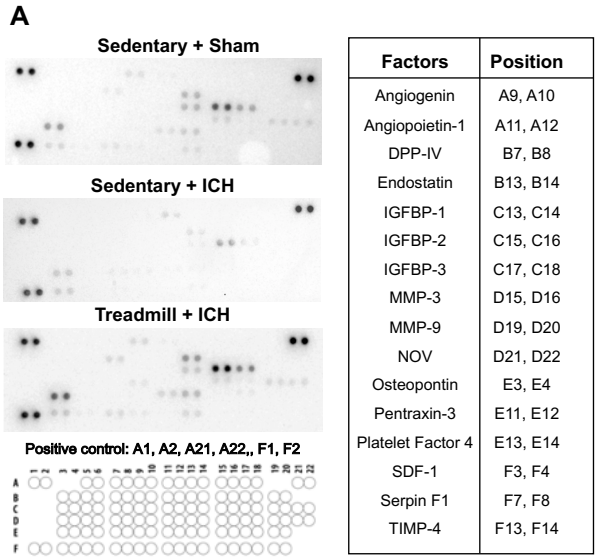
Immunohistochemistry - At 8 days after ICH induction, mice were anesthetized and were transcardially perfused with 0.9% physiological saline and 4% paraformaldehyde. Then, brains were isolated and preserved in 4% paraformaldehyde overnight at 4℃ and soaked in 30% sucrose overnight at 4℃. Five samples were randomly selected from each group, and 20 μm-thick coronal sections were prepared for immunostaining using cryostat CM1520. After being rinsed with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBS/T) for 5 minutes 3 times, sections were incubated in PBS/0.3% BSA solution for 1 hour at room temperature. Then, sections were incubated in PBS/0.3% BSA solution containing primary antibodies overnight at 4℃. For detection of basement membrane on blood vessels, astrocytes, microglia/macrophages and CD36, goat anti-type IV collagen-UNLB (1:200; cat# 1340-01,

SouthernBiotech, USA), glial fibrillary acidic protein (GFAP) monoclonal antibody (rat) (1:200; cat# 13-0300, Thermo Fisher scientific, IL, USA), rabbit anti-IBA1 antibody (1:200; cat# 019-19,741, FUJIFILM Wako Pure Chemical, Osaka, Japan), and anti-CD36, Clone:FA6.152 (mouse) (1:200; cat# COIM0765, Beckman Coulter™, France) were used as primary antibodies. After being rinsed with PBS/T for 5 minutes 3 times, sections were incubated with the corresponding secondary antibodies (1:1000, Jackson Immunoresearch Laboratories) for 2 hours at room temperature. Finally, the sections were washed three times with PBS and covered with VECTASHIELD® mounting medium with DAPI (Vector Laboratories, USA). Stained sections were observed with ECLIPSE Ti-S (Nikon) and scanned with the Retiga™ 2000R Fast 1394 Digital Camera. All brain sections were blinded to the examiner before evaluation of fluorescence intensity. To calculate collagen type IV and GFAP positive area, the number of Iba1 positive cells, and the number of Iba1, CD36 and DAPI triple-positive cells in the peri-hematomal region, the bilateral side of the hematoma in two coronal sections were evaluated. Then, we set three regions of interest by drawing 200 × 200 μm^2 squares for collagen type IV and GFAP, and 100 × 100 μm^2 squares for Iba1 and Iba1, CD36 and DAPI. Next, for Iba1 and Iba1, CD36 and DAPI, the number of cells per mm^2 was calculated, and the average of 6 areas was calculated per mouse.

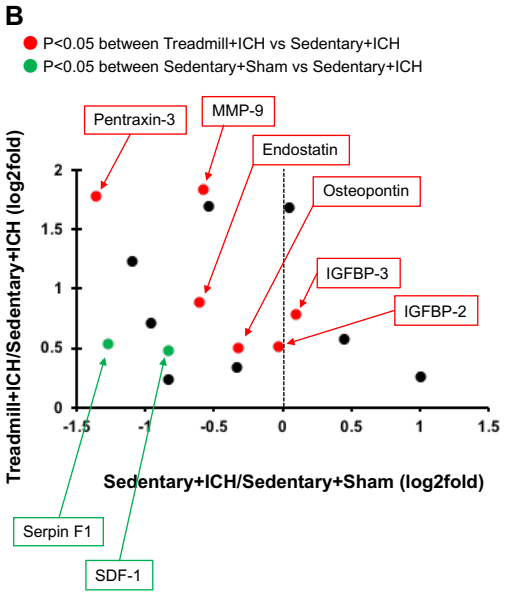
Plasma sample preparation and protein array experiment - At 8 days after ICH induction, blood was collected from the inferior vena cava under anesthesia and was mixed with EDTA. Then, the plasma was separated by centrifuging blood at 1,200 g for 30 min at 4℃. Plasma samples were stored at -80℃ for later array analysis. Soluble factors in plasma were measured by using the Proteome Profiler Mouse Angiogenesis Array Kit (R&D System, USA) according to the manufacturer's instruments. Optical density of each signal was measured using Image-J software and calculated based on the positive control. To conduct protein array experiments, we randomly selected 8 animals for each group. One animal in the Sedentary+Sham group was an outlier for most factors and was thus excluded from the analysis.

Statistical Methods - All measurements/analyses were conducted by operators who were blinded to the group allocations. Statistical analysis was conducted by unpaired t-test, one or two-way repeated-measures analysis of variance, followed by Sidak or Tukey's multiple comparisons test. Differences with $P < 0.05$ were considered statistically significant, and data were expressed as mean plus and/or minus SD.

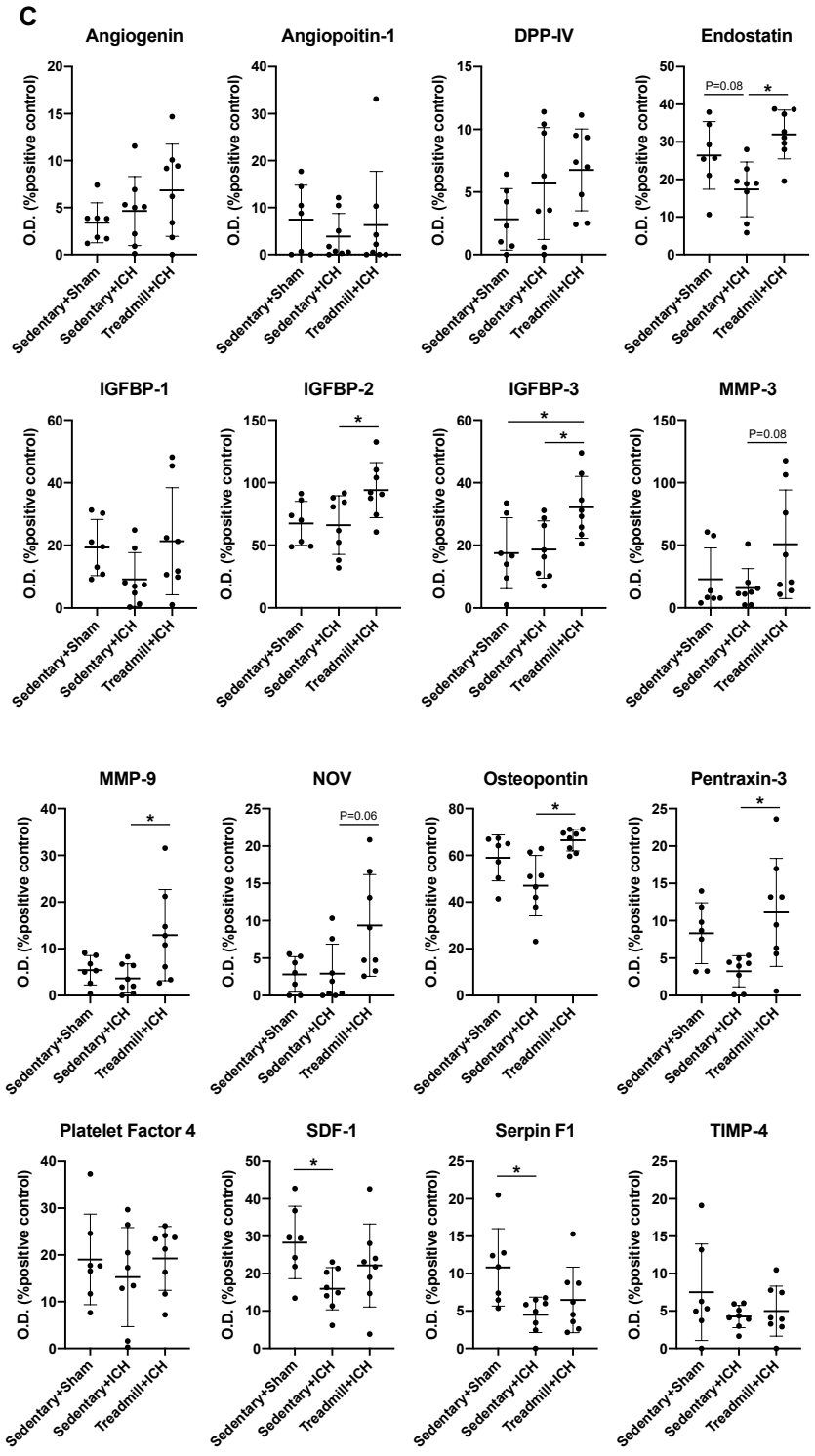
Supplementary Figure I: Full data set of protein array experiments using mouse plasma samples. Mouse plasma samples were prepared at day 8 after ICH induction. **(A)** Representative images of protein array membranes and the list of detected factors in our study. **(B)** Summary of quantitative results for the 16 detected factors. X-axis shows the ratio of "Sedentary+ICH/Sedentary+Sham (log2fold)", and Y-axis is for "Treadmill+ICH/Sedentary+ICH (log2fold)". Each dot represents one factor. The red dots are factors that showed significant differences between Treadmill+ICH vs Sedentary+ICH, and the green dots are factors that showed significant differences between Sedentary+Sham vs Sedentary+ICH. The black dots are other factors, which did not show any significant differences between the groups. It was noted that the average values of all 16 factors in ICH mice with treadmill preconditioning were larger than the ones in ICH mice without exercise. **(C; please see next page)** Quantitative results of the detected factors in our array experiments. Data are mean ± SD. *P<0.05. For statistical analysis, multiple group comparisons were performed by One-way ANOVA – by post-hoc Tukey test for groups of normal distribution, or Kruskal-Wallis followed by post-hoc Dunn's test for groups of non-normal distribution. The Shapiro-Wilk test was used to check the distribution of our data.



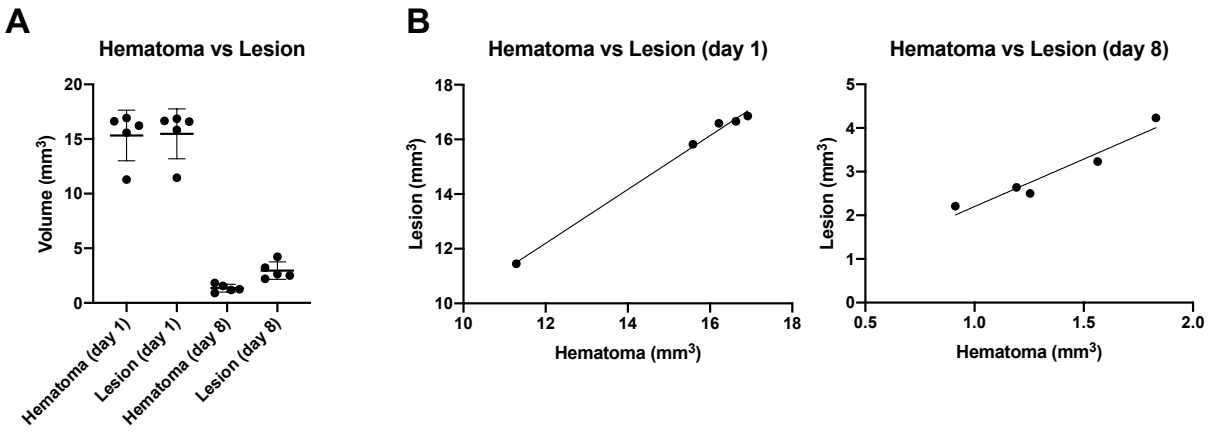
Factors	Position
Angiogenin	A9, A10
Angiopoietin-1	A11, A12
DPP-IV	B7, B8
Endostatin	B13, B14
IGFBP-1	C13, C14
IGFBP-2	C15, C16
IGFBP-3	C17, C18
MMP-3	D15, D16
MMP-9	D19, D20
NOV	D21, D22
Osteopontin	E3, E4
Pentraxin-3	E11, E12
Platelet Factor 4	E13, E14
SDF-1	F3, F4
Serpin F1	F7, F8
TIMP-4	F13, F14



Supplementary Figure I (continued)



Supplementary Figure II: Comparison between hematoma volumes vs lesion volumes in our mouse ICH model. Both hematoma and lesion volumes decreased from day 1 to day 8 **(A)**, and the hematoma volume showed a correlation with the lesion volume ($R=0.9974$, $p < 0.05$, for day 1; $R=0.9612$, $p < 0.05$, for day 8) **(B)**. Data are mean ± SD.



*** 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:	N/A
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:	N/A

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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