

Supplementary methods

Animals

For this study 84 spontaneously hypertensive rats (SHRs) were used (10 weeks old, male, body weight approximately 260 g; Charles River Laboratories, Sulzbach, Germany). SHR constitute a well-established hypertensive rat model.^{1,2} All experimental procedures were conducted in accordance with the National Institutes of Health's guide for the care and use of laboratory animals. The report of the data in this manuscript is compliant with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.³ This study was approved from the responsible regulatory authorities in Germany (Regierungspraesidium Darmstadt, approval number FU/1070).

Study design and sample size calculation

Previous studies showed a significantly worse functional outcome for hypertensive vs. normotensive rats when intracerebral hemorrhage (ICH) was induced.⁴ Thus, we assumed a beneficial effect of an intensive blood pressure lowering strategy after ICH. Rats were randomized into an interventional (intensive blood pressure lowering) group and a control group, respectively. Besides anti-hypertensive treatment, both groups were treated identically. Hematoma volume and neurological deficits were determined 24 hours after ICH induction (time point of expected maximum hemorrhage expansion and neurological impairment). Histopathological analysis and magnetic resonance imaging (MRI) were performed 24 and 72 hours after ICH induction (to enhance sensitivity for secondary injury after ICH during the first 3 days). An overview of animal numbers per time point can be found in the Supplementary Table 1.

Based on the effect size of our previous studies (Cohens $D=1.75$),⁵ we needed at least seven rats per group in order to detect a significant difference in hematoma volume between the treatment regimens with a power of 80% and a type 1 error of 0.05 using a two-tailed Student's *t*-test. Thus, we randomized nine rats per group.

ICH induction

All surgery was performed under isoflurane anesthesia (1.5% to 2%) with spontaneous respiration. For analgesia, 0.1 mg/kg buprenorphine were applied. For ICH induction, a small borehole was drilled and a 30-gauge 5 μ L-microinjection needle (Hamilton, Bonaduz, Switzerland; 75RN series) was slowly lowered into the right striatum at the following coordinates from bregma: 0.2 mm anterior, 3.5 mm lateral, and 5.5 mm depth. A 2 μ L of saline containing 0.25 U collagenase VII-S (Sigma,

Darmstadt, Germany) or saline alone were injected over 5 minutes using a microinfusion pump (Quintessential Stereotaxic Injector, Stoelting Co., Wood Dale, IL, USA). The needle was left in place for 10 minutes and was then slowly removed. Afterwards, the borehole was sealed with bone wax, the scalp was closed, and the rats were allowed to recover. The duration of the whole surgical procedure was approximately 35 minutes for each rat. A closed-loop temperature system with a heating pad and a rectal temperature probe was used to maintain body temperature.

Blood pressure lowering in acute ICH

Intensive blood pressure management in acute ICH requires specific qualities for the drug of choice: (1) orally applicable to avoid stress induced hypertension; (2) quickly effective to ensure rapid blood pressure lowering after ICH induction; (3) non-sedating to allow neurological monitoring. Unlike clonidine, nifedipine fulfils these criteria without relevant side effects. Nifedipine is a dihydropyridine calcium channel blocker. The anti-hypertensive effects of nifedipine in rats administered by chow for up to 20 weeks are well characterized, no interaction with collagenase VII-S is known.⁶

The intensive blood pressure lowering group received nifedipine (oral liquid, 4 mg/mL, Ratiopharm, Ulm, Germany) per nasogastric tube. Based on the results of explorative tests, a dosage of 15 mg/kg body weight with an application volume of 0.2 mL per treatment was chosen. In the control group saline alone was administered in the same frequency and volume. The first oral administration of nifedipine or saline, respectively, was scheduled at 30 minutes after ICH induction, to ensure the swallowing reflex. Thereafter, rats were fed every 8 hours during the first 24 hours, then every 12 hours.

Arterial blood pressure was measured non-invasively using a photoelectric tail cuff device (BP 2000, blood pressure system for rats, Biomedical Instruments, Zöllnitz, Germany). This method has previously been validated in SHR.^{2,7} Rats were placed in a restrainer on a heating plate with a temperature of 36°C. A cuff was attached to the tail and gently tightened until detection of the pulse signal. The animals acclimatized on the plate for 10 minutes, followed by 10 pre-measurements to reduce stress level. Ten measurements were then performed to generate mean values for systolic and diastolic blood pressure. To avoid any impact of tail cuff measurements during critical postoperative hours, blood pressure was not evaluated in animals scheduled for hematoma quantification, histopathological analysis or outcome assessment. Instead, we analyzed separate animals after nifedipine/saline treatment up to 72 hours to en-

sure stable blood pressure lowering in the intervention group.

Functional outcome

Neurological deficits were evaluated by a blinded rater 24 hours after ICH induction using the modified Neurological Severity Score (mNSS).^{8,9} The 18-point mNSS includes testing for hemiparesis, gait, coordination and sensory functions. Videotaped sequences were assessed for spontaneous motion activity and the beam walking test, both parts of the mNSS. Rats were not trained before the tests. Rats that died within the observation period were assigned the maximum of 18 points.

Determination of ICH volume

After mNSS assessment, rats were transcatheterially perfused with 50 mL phosphate buffered saline (PBS) under deep isoflurane anesthesia. Hemoglobin concentration was measured for each hemisphere separately following a previously described protocol.⁵ In brief, hemispheres were homogenized, subjected to ultrasound for 60 seconds and centrifuged (13,000 rpm, 4°C, 30 minutes). Photometric analysis of the supernatant mixed with Drabkin's Reagent solution (Sigma-Aldrich, Taufkirchen, Germany) was then performed in duplicates at 540 nm. ICH volume was calculated based on a standard curve (data not shown in detail).

Rats found dead within the observation period could not undergo transcatheterial perfusion. In this case, we performed a total autopsy to exclude extracerebral bleeding. Afterwards, we determined ICH volume as described above and subtracted 2.4 µL. This value was found to be the estimated cerebral blood volume in SHR.¹⁰

Histopathological analysis

After transcatheterial perfusion with 40 mL PBS and 40 mL buffered formalin (pH 7.4), rat brains were stored in buffered formalin and embedded in paraffin. Fixed brains were cut through the needle entry site (identifiable on the brain surface) as well as stepwise every 1 mm anterior and posterior to that plane. Sections (3 µm) were obtained using a microtome (Quintessential Stereotaxic Injector, Stoelting), placed on SuperFrost Plus slides, and deparaffinized. Hematoxylin eosin (HE) staining was performed. After incubation, slides were mounted in isopropanol followed by xylene.

Immunohistochemical analysis was performed on the Discovery XT Immunohistochemistry System (Ventana, Strasbourg, France) as previously described.¹¹ The following anti-rat antibodies were applied: rabbit anti-immunoglobulin G (IgG) (H&L, ab6703, dilution 1:2,000, Abcam, Cambridge, UK), and mouse anti-major histocompatibility complex class II antibody (MRC

OX-6, ab23990, dilution 1:500, Abcam). Sections were washed, counterstained with hematoxylin and bluing reagent and mounted. Evaluation of the stainings and photographic documentation were performed using an Olympus BX-50 light microscope (Hamburg, Germany).

Ordinal histoscores were calculated using a semi-quantitative assessment for edema formation (HE staining, vacuolated tissue compatible with vasogenic brain edema: 0=isolated hemorrhage, edema absent; 1=edema minimal, no midline shift; 2=edema minimal to medium with cortical involvement, no midline shift; 3=edema medium to strong with midline shift; 4=strong edema bilateral, midline shift) and blood brain barrier dysfunction (IgG staining, IgG extravasation as indirect marker of blood brain barrier dysfunction: 0=isolated hemorrhage, extravasation absent; 1=extravasation and blood-brain-barrier breakdown minimal; 2=extravasation minimal to medium; 3=extravasation medium to strong; 4=extravasation strong and bilateral). Six slides per brain prepared from needle entry site ± 1 mm were analyzed. The slides were evaluated in blinded random order with a standard light microscopy. Median scores were calculated.¹²

Post-mortem MRI

Post-mortem MRI was applied as described elsewhere.¹³ Following an identical experimental procedure as described above, 40 mL warmed saline-Prohance (in 0.9% saline, 37°C, 1:10 solution of Prohance, Bracco, Princeton, NJ, USA) was used for perfusion (4 minutes). Then 40 mL formalin-Prohance (in 10% formalin, 1:10 solution of Prohance, Milano, Italy) was given for another 4 minutes. Whole rat heads were removed and put into 10% formalin for 24 hours without Prohance. Heads were then transferred into Prohance-PBS (1:100 solution) and stored at 4°C. MRI was performed using a 7 Tesla small animal scanner (PharmaScan, Bruker, Ettlingen, Germany) with a volume coil. Data acquisition was performed using the Paravision 6.0.1 software (Bruker, Billerica, MA, USA). Modified driven equilibrium Fourier transform (MDEFT) sequence was used for T1-weighted images with the following parameters: echo time (TE)=2 ms, relaxation time (TR)=4,000 ms. The matrix size was 256x256 and the voxel size 0.17 mm. T2 sequences were measured afterwards (TR=2,200 ms; TE=33 ms). The matrix size was 512x512 and the voxel size 0.08 mm. Total imaging time was 3 hours per rat head. Data analysis was performed by a blinded rater slide by slide quantifying hematoma and edema volume on T1 and T2-weighted images (MRIcro, Chris Rorden, Columbia, SC, USA; www.mricro.com).

Statistical analysis

Prism 7 (GraphPad Software, San Diego, CA, USA) and SPSS version 22.0 (IBM Co., Armonk, NY, USA) were used for statistical analysis. Blood pressure levels are presented as mean±standard deviation (unless otherwise stated, systolic blood pressure values are mentioned). Hematoma volume results are displayed in a scatter dot plot with mean values. Statistical significance was assessed using the two-tailed t-test after passing Shapiro-Wilk test. Neuroscore data are displayed in scatter plots with median values and interquartile range (IQR). Statistical significance was assessed using the Mann-Whitney U-test. For histoscore data medians and IQR are given and statistical significance was assessed using the Mann-Whitney U-test. Statistical significance was set to $P<0.05$.

Supplementary References

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