Supplemental Material

Microthrombi Correlates with Infarction and Delayed Neurological Deficits After Subarachnoid Hemorrhage in Mice

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Running title: Delayed deficits after SAH in mice

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Supplemental Methods

Adult C57BL/6J mice (4-6 months old) were used in all experiments. Twenty-four male and 22 female mice were used in preliminary experiments. Based on our preliminary findings, sample size calculation suggested that 38 male (8 sham, 30 SAH) and 28 female (8 sham, 20 SAH) mice were needed to validate our preliminary findings. Animals were housed in a humidity- and temperature-controlled room with a 12 hour light-dark cycle, and given ad libitum access to food and water. Mice were electronically randomized into either the sham or SAH group according to sample size calculations. The same surgeon performed SAH and sham surgeries and all animals were treated with the same amount of buprenorphine and saline (on the day of surgery). All investigators responsible for functional assessment, measurement of outcomes, and data analysis were blinded to sex and experimental groups.

Subarachnoid hemorrhage was induced via endovascular perforation in mice as previously described.[1] Briefly, mice were anesthetized with isoflurane (5% induction, 1.5-2.5% maintained). Buprenorphine was subcutaneously injected (0.05-0.1 mg/kg). The animal was placed supine, then the surgical site was shaved, bupivacaine (2 mg/kg, subcutaneous) was given, and the surgical site was sterilized with betadine and 70% ethanol. A vertical midline incision was made in the neck and the external carotid and common carotid arteries were isolated. The external carotid artery was ligated, leaving a stump, and then the internal carotid artery was isolated. Mice were then endotracheally intubated using the plastic cannula of an intravenous 20 Gauge catheter. Vessel clips were placed on the common carotid and internal carotid arteries to

momentarily stop blood flow. A cut was made in the external carotid artery stump and a 5-0 monofilament nylon suture was inserted. The vessel clip on the internal carotid artery was removed and the suture was advanced until vessel perforation (approximately 11 mm). The suture was not inserted more than 12 mm to prevent advancing the suture too far. The suture was withdrawn and the neck incision was sutured. Immediately following perforation, breathing rate becomes irregular and typically ceases for 5-30 seconds[2-4] (Table I). Mice which were not breathing 1.5 minutes after perforation were then connected to a mechanical ventilator and ventilated for 5-10 minutes (RoVent Jr., Kent Scientific). When the animal was able to breath without mechanical support, it was allowed to recover. Afterwards, mice were placed back into their home cages and housed in groups of 1-5 mice per cage. Analgesic and saline were given BID for 3 days as necessary for mice subjected to SAH. Animals allocated into the sham group underwent all the same surgical procedures.

Mice were allowed to survive for up to 7 days after SAH. Animals were assessed a minimum of three times per day for health status by our lab and UTHealth Vet staff. Mice which were determined unable to survive overnight were euthanized and brains were collected for post-mortem analysis of vasospasm and microthrombi.

Behavioral performance was assessed every day from days 1-7 post-SAH using an 8-test neuroscore which evaluates functional performance in exploration, climbing, forelimb and hindlimb use, whisker and side sensation, balance, and visual reflex.[1] Table II shows the specifics of the scoring criteria for the neuroscore.

Five minutes before euthanasia, mice received a subcutaneous injection of heparin (5,000 Units/kg) to minimize the development of new thrombi which may occur during perfusion.[5, 6] At the time of euthanasia or 7 days post-SAH, mice (n=4-5/group) underwent transcardial perfusion of PBS, then DiI (12μg/mL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), followed by 4% PFA at 80-100 mmHg.[6] The remaining mice in each group did not have DiI perfused. Brains were removed and stored in 4% PFA overnight, then PBS until vibratome sectioning. Prior to vibratome section, the circle of Willis was imaged in DiI stained brains using Leica M205FA stereo-fluorescence microscope. These images were used to quantify large artery diameters (the surrogate marker for vasospasm). Vessel painting has been previously shown to yield vessel diameters similar to those measured using the gelatin-India ink method.[5] Figure I is a schematic of mouse usage and allocation into the various endpoints.

We allocated 5 mice per group for vessel painting with DiI to quantify the large arter diameters to assess vasospasm. Our sample size calculation used the data provided by Aum *et al.*[5] which had a mean difference of 25 and a SD of 8 (Figure 4 of the article by Aum *et al.*[5]). Using this mean difference and SD, for a power of 0.8 and an alpha of 0.05, Sigmaplot estimates that 4 mice per group is sufficient to test for a statistical difference among 3 groups. We allocated 5 mice per group so account for any potential mortalities (of which we had 1 Male SAH mouse) (Figure I).

Table I. Respiration Changes Observed from Suture Insertion until Suture Removal. No sham mouse showed any respiratory changes or distress. SAH mice which developed delayed deficits are highlighted in grey.

SAH Males		SAH Females	
Mouse	Respiration Changes	Mouse	Respiration Changes
1	Irregular for ~5sec	1	Apnea for ~45sec
2	Apnea for ~30sec	2	Apnea for ~40sec
3	Apnea for ~20sec	3	Irregular for ~30sec
4	Irregular for ~10sec	4	Irregular for ~15sec
5	Irregular for ~15sec	5	Apnea for ~30sec
6	Apnea for ~60sec	6	Apnea for ~15sec
7	Apnea for ~10sec	7	Apnea for 90sec
8	Apnea for ~15sec	8	Irregular for ~5sec
9	Apnea for ~15sec	9	Apnea for ~30sec
10	Irregular for ~30sec	10	Irregular for ~40sec
11	Irregular for ~10sec	11	Apnea for 90sec
12	Irregular for ~10sec	12	Apnea for ~30sec
13	Irregular for ~60sec	13	Apnea for ~30sec
14	Irregular for ~5sec	14	Irregular for ~10sec
15	Irregular for ~30sec	15	Irregular for ~60sec
16	Apnea for ~45sec	16	Irregular for ~15sec
17	Apnea for 90sec	17	Normal Respiration
18	Irregular for ~10sec	18	Apnea for ~10sec
19	Died from apnea	19	Irregular for ~10sec
20	Apnea for ~40sec	20	Irregular for ~30sec
21	Irregular for ~15sec		
22	Irregular for ~40sec		
23	Irregular for ~15sec		
24	Irregular for ~5sec		
25	Apnea for ~60sec		
26	Apnea for ~45sec		
27	Normal Respiration		
28	Apnea for ~10sec		
29	Irregular for ~5sec		
30	Normal Respiration		

Table II. Neuroscore for Evaluating Functional Deficits after Subarachnoid Hemorrhage in Mice. Score range is from 0 (maximum deficits) to 24 (no deficits).

Sub-Test	**0**	**1**	**2**	**3**
Spontaneous Activity (3 min)	No Movement	Minimal Movement	Touches 1-2 Walls	Touches 3-4 Walls While Standing on Hind-Limbs
Climbing (1 min)	Weak Grip and Fall Down	Climbs but Doesn't Reach Top and Weak Grip	Climbs to Top and Weak Grip or Doesn't Climb to Top and Good Grip	Climbs to Top and Strong Grip
Balance	Tumbles	Stands, Sways	Sways as Walking	Changes Position Easily
Side Stroking	No Response	Unilateral Response	Bilateral Weak Response or Strong Ipsilateral Response	Strong Bilateral Response
Vibrissae Touch (stroke whiskers)	No Response	Unilateral Response	Weak Bilateral Response	Strong Bilateral Response
Visual (tip toward each eye)	No Response	Unilateral Response	Weak Bilateral Response	Strong/Rapid Bilateral Response
Forelimb Use	Severe Bilateral Deficits	Severe Unilateral Deficits	Slight Deficits (Unilateral or Bilateral)	None
Hindlimb Use	Severe Bilateral Deficits	Severe Unilateral Deficits	Slight Deficits (Unilateral or Bilateral)	None

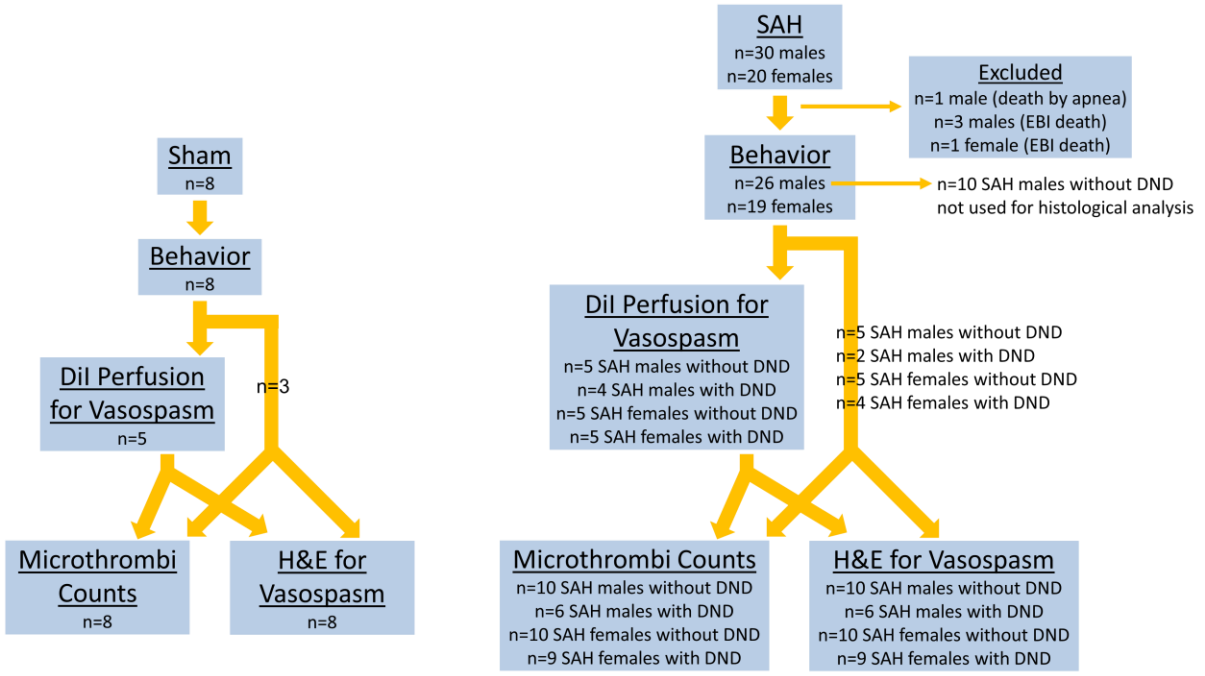
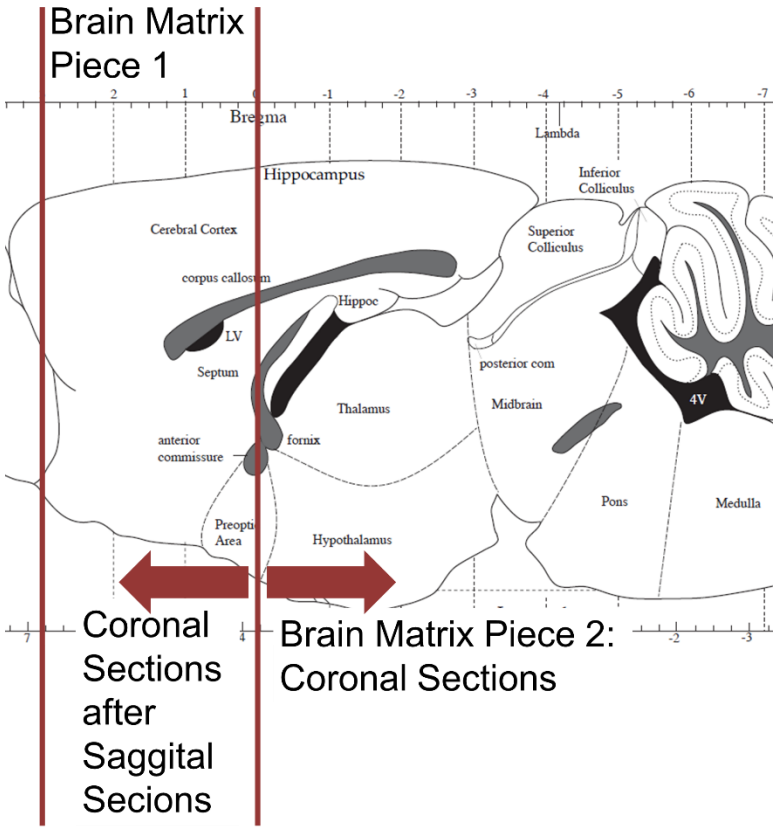


Figure I. Schematic of Sample Allocation into the Various Endpoints. No sham died, nor did any Sham get delayed deficits. Four males (one died immediately after perforation, three died overnight after surgery) and one female (died 6 hours post-SAH) were excluded. For the microthrombi counts and H&E vasospasm analysis, 10 male and 10 female mice which did not develop DND were used, and all mice which developed DND were used (n=6 males and n=9 females). EBI: early brain injury.

The ACA and MCA territories were sectioned into 40 μm thick brain slices following the method of Wang *et al.*[4] After removal of the brain post-perfusion, we used a brain matrix (Kent Scientific) to separate the brain into two pieces at bregma. The first piece was the anterior portion of the brain which had the anterior and middle cerebral arteries. The second piece was the remaining portion of the brain which was coronally-sectioned into 40 μm thick slices. The first piece was embedded into the agar mold, and sagittal sections (sixty 40 μm thick slices were taken to visualize the middle cerebral artery) were taken from one side. The brain was then re-positioned so sagittal sections (sixty 40 μm thick slices were taken to visualize the middle cerebral artery) could be taken from the other side of the brain. After saggitally-sectioning each side of the anterior piece of the brain, the brain was re-positioned so the remaining piece could be coronally-sectioned (40 μm thick slices to visualize the anterior cerebral arteries). See Figure II for anatomical details.

Brain sections were used to assess vasospasm (a secondary method for measuring large artery diameters) and microthrombi. Mice which underwent DiI staining for vessel diameters also were used to assess microthrombi and large artery diameters (using H&E). Vasospasm was assessed using H&E stained sagittal sections for MCA measurement, and coronal sections were used for measurement of the ACA (+2.5 to 0 mm from bregma), ICA (0 to -3 from bregma), and BA (-5.5 to -8 from bregma).



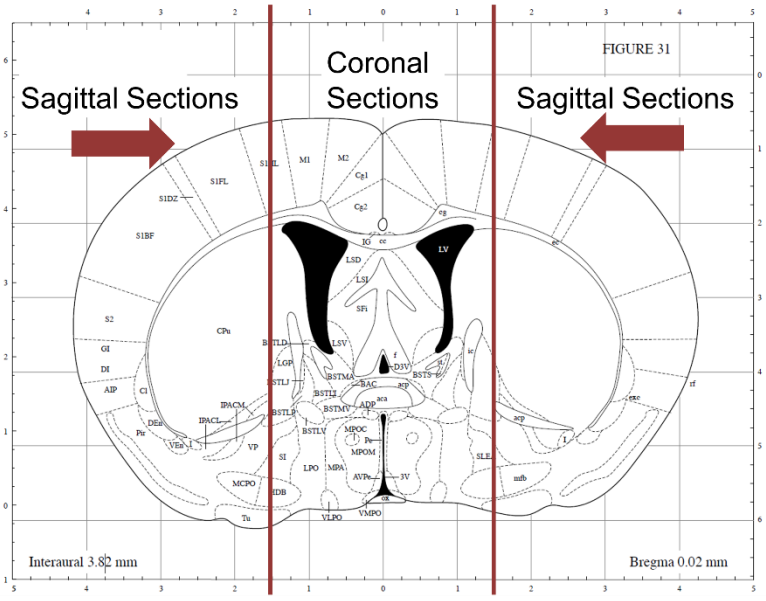


Figure II. Mouse Brain Stereotaxic Atlas images of the way the brains were sectioned for histological analysis of large cerebral arteries and microthrombi number.

Microthrombi counting was performed in coronal sections at -2 from bregma[4] using two staining methods: H&E staining and Martius, Scarlet, and Blue (MSB) staining. Both staining methods are conventional methods for assessing thrombi.[7-10] The mice which were used for vasospasm analysis were also used for microthrombi counts and infarction.

All tests were two sided. Data is presented as individual data points or mean with standard deviation. Mice dying from early brain injury (0-48 hours post-SAH) were excluded from analysis. Nonparametric analysis of longitudinal neurobehavior data was performed using a F1-LD-F1 design in nparLD R software package. Vasospasm and microthrombi count were analyzed using one-way ANOVAs with Tukey's HSD post-hoc test. Survival analysis was performed to analyze the time to DND event data. The survival time was calculated from the day of perforation to the date of DND or the last follow up (*i.e.* day 7). A Kaplan-Meier survival curve was used to plot the time to DND and the statistical significance was analyzed by log-rank test. Correlation was computed using a point-biserial correlation coefficient. GraphPad Prism 6 (La Jolla, CA, USA) was used for analysis. We confirmed all statistical analyses with SigmaPlot v11.0.

Microthrombi were counted for the entire coronal section at -2 from bregma[4] using two staining methods: H&E staining and Martius, Scarlet, and Blue (MSB) staining. Both staining methods are conventional methods for assessing thrombi.[7-10] Microthrombi counting was performed by the same individual for all brains. MSB stained slices were observed at 20x and every microthrombi (identified by red staining within the vasculature) was counted throughout the entire slice (-2 from bregma). The lengths of the microthrombi were not quantified; indeed some microthrombi are longer than others. While longer microthrombi may be more deleterious to the tissue, the reason count was used rather than length is because some vessels were in-plane, while others were perpendicular to the plane. Thus, we thought that assessing length would introduce artifacts if one brain slice had more in-plane vessels versus another slice.

Infarct volume was assessed in MSB-stained brain slices every mm from +2 to -3 from Bregma. For each slice, an investigator blinded to experimental group and sex identified the infarct area(s) (mm^2). After all six slices was assessed, the areas within each slice were summed and multiplied by the distance between the slices to obtain the infarct volume.

All tests were two sided. Data is presented as individual data points or mean with standard deviation. Mice dying from early brain injury (0-48 hours post-SAH) were excluded from analysis. Nonparametric analysis of longitudinal neurobehavior data was performed using a F1-LD-F1 design in nparLD R software package. Vasospasm and microthrombi count were analyzed using one-way ANOVAs with Tukey's HSD post-hoc test. Survival analysis was performed to analyze the time to DND event data. The survival time was calculated from the day of perforation to the date of DND or the last follow up (*i.e.* day 7). A Kaplan-Meier survival curve was used to plot the time to DND and the statistical significance was analyzed by log-rank test. Correlations were computed using point-biserial correlation coefficient (for correlation with DND) or Pearson correlation coefficients (for correlations among microthrombi count, large artery diameters, and infarct volume). GraphPad Prism 6 (La Jolla, CA, USA) was used for analysis. We confirmed all statistical analyses with SigmaPlot v11.0.

Supplemental Results and Discussion

Preliminary Findings on Delayed Deficits

In our preliminary studies, we observed that mice with SAH recovered from their day 1 functional deficits (as expected), but a small subset of mice would experience a drastic decline in functional performance during days 3-5 post-SAH. Indeed, a number of these mice would die during this delayed time period despite having near-perfect functional performances the day before. In our preliminary study, we observed 4/24 males (17%) and 8/22 females (36%) developed delayed deficits 3-5 days post-SAH. Although at the time we did were not sure if a sex difference was real (odds ratio was 2.18), we realized that what we were observing was similar to the delayed neurological decline described in humans suffering from SAH. Thus, the current study was designed to validate our preliminary findings. Before doing so, we had to define delayed deficits in mice. We used a composite neuroscoring system which is sensitive to SAH injury.[1] Using the data from the preliminary dataset of 24 males and 22 females, we examined the receiver operating characteristic (ROC) curves for each subtest of the composite neuroscore (Table III). The motor subtests (spontaneous activity, climbing, balance, forelimb use, and hindlimb use) are able to identify mice with DND from mice which do not develop DND; the three reflex subtests (side stroking vibrissae touch, and visual reflex) were unable to distinguish DND mice from mice without DND. We confirmed this analysis using the cohorts described within this manuscript and got results which had the same statistical meaning. Based on the ROC analysis, we defined delayed neurological deficits as a drop of 5 or more points within the motor subtests (spontaneous activity, climbing, balance, forelimb use, and hindlimb use) after a recovery of some function from post-SAH day 1. Mice must have some functional recovery prior to declining to be classified as DND. If no recovery occurs, then the mouse may have just declined due to early brain injury or some other cause.

Table III. Receiver Operating Characteristic (ROC) Curve Analysis for the Individual Sub-Tests for SAH Mice With and Without DND. Bold fond indicates statistical significance after correcting for multiple comparisons with the Bonferroni method (*i.e.* p<0.00625 is statistically significant). AUC: area under the curve.

	AUC (95% Confidence Interval)	p-Value
Spontaneous Activity	0.9643 (0.8843 to 1.044)	**<0.0001**
Climbing	0.9578 (0.8750 to 1.041)	**<0.0001**
Balance	0.8929 (0.7605 to 1.025)	**<0.0001**
Forelimb Use	0.9286 (0.8179 to 1.039)	**<0.0001**
Hindlimb Use	0.9643 (0.8843 to 1.044)	**<0.0001**
Side Stroking	0.6948 (0.5121 to 0.8775)	0.0516
Vibrissae Touch	0.6883 (0.4993 to 0.8773)	0.0599
Visual Reflex	0.6948 (0.5121 to 0.8775)	0.0516

Current Findings

Of mice receiving SAH, one male and one female were excluded due to hemiparesis which was observed upon recovery from anesthesia following SAH surgery. These mice were replaced.

Six of twenty-six (23%) male mice subjected to SAH developed DND on days 3 (n=1), 5 (n=3), and 6 (n=2) post-SAH. Nine of nineteen (47%) female SAH mice got DND on days 3-6 following SAH; three on day 3, one on day 4, six on day 5, and one on day 6. Two of the females which developed DND on day 3 recovered before deteriorating again (Figure 1, Tables IV and V).

Since in both of our cohorts for delayed deficits showed that females experienced delayed deficits at higher odds ratios, we tested the time to delayed deficits for statistical differences between males and females. Female mice have a statistically significant faster time to delayed deficits than male mice (Figure 1C), suggesting that female sex may be a risk factor for experiencing DND. This finding in mice is also observed in human SAH patients.[11]

When testing for the group effect in neurobehavioral performance, there are significant differences in functional performance between sham and SAH mice on days 1-3, but due to the ability of mice to spontaneously recover,[12] no significance is observed at later time-points (days 4-7 for males, days 6 and 7 for females) (Figure III). Overall injury effect (*i.e.* sham vs SAH performance) is statistically significant for both sexes ($p<0.0001$ for males, $p<0.0001$ for females).

To confirm our microthrombi counts, we performed 4 separate methods for viewing microthrombi. First, we used a specific staining method which is able to differentiate red blood cells from a thrombi; the MSB staining method (Figure 2) stains red blood cells yellow while fibrin stains red (Figure IV).[7-10] Second, we used H&E staining which has been widely used for microthrombi assessment.[4] Our counts from the MSB and H&E stained brains is presented in Tables VI-VIII. Our counts from H&E stained brain sections and MSB stained sections were similar with MSB staining counts being slightly higher in SAH mice with DND and slightly lower in sham mice (Table VI-VIII). In fact, it seems that MSB staining may be a better method than H&E since microthrombi are more clearly distinguished from the surrounding tissue (see Figures 2, IV and V). Thus, the microthrombi counts from MSB stained brain slices are reported in Figure 2 and were used for statistical and correlation analysis.

One concern with detecting microthrombi using H&E staining is whether or not the Eosin stain is showing a thrombi or erythrocytes. While perfusion should remove all erythrocytes from the vasculature, there is the possibility of poor/incomplete perfusion, especially within the microvasculature. Since the MSB stain can differentiate erythrocytes from thrombi (Figure IV), it seems to be a better stain than H&E for thrombi within the brain; the MSB stain provides more confidence in microthrombi counts and removes potential artifacts from poorly/incompletely perfused blood vessels. See Figures IV and V for representative images for each of the two staining methods.

No statistical differences were observed for the microthrombi counts of male versus female shams ($p=0.8424$), male versus female SAH mice without DND ($p=0.5419$), or male versus female SAH mice with DND ($p=0.2870$).

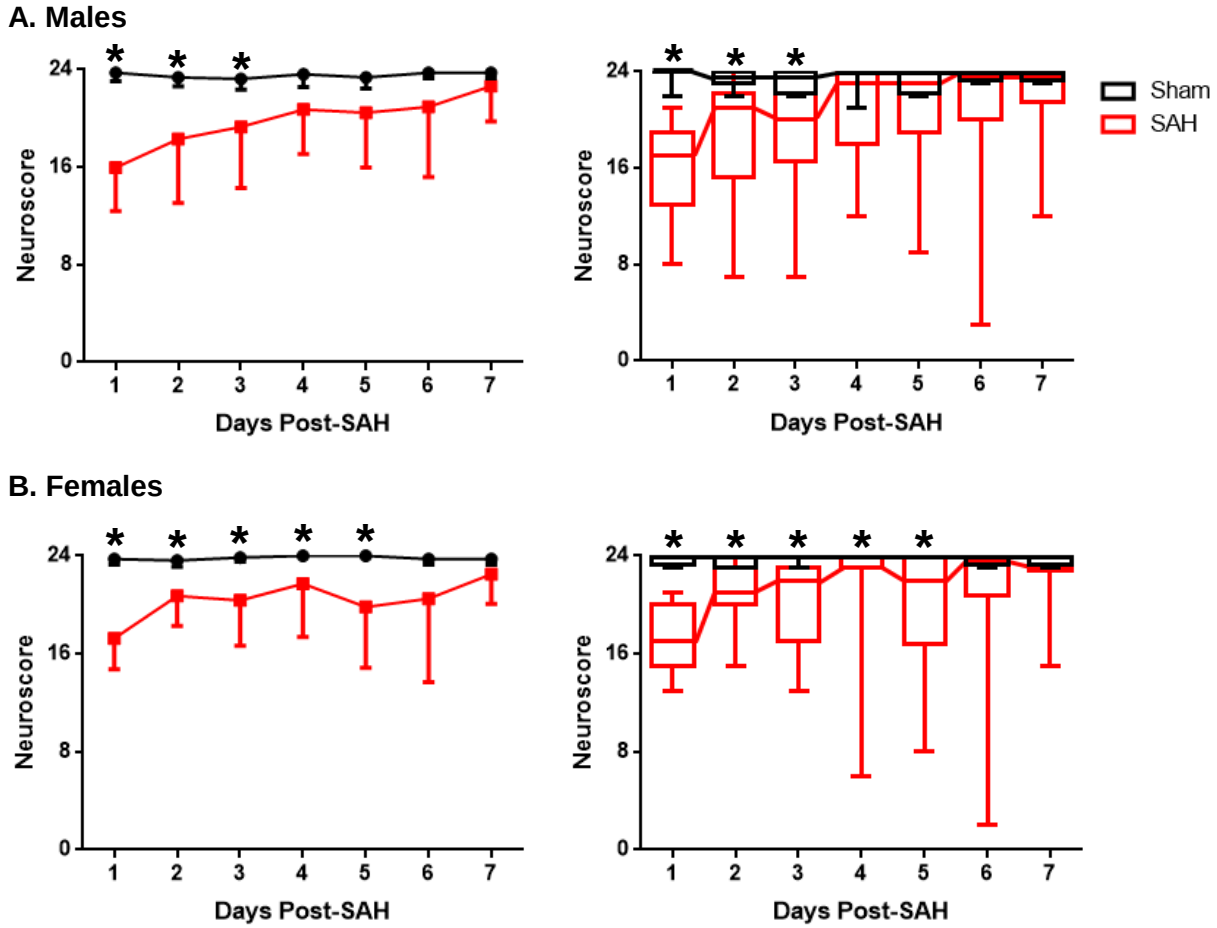


Figure III. Neuroscore Performance of Mice after SAH. **A.** Male Mice (Sham: n=8 on all days; SAH: n=26 Days 1-2, n=25 Day 3, n=23 Days 4-5, n=22 Day6, and n=20 Day 7). **B.** Female Mice (Sham: n=8 on all days; SAH: n=19 Days 1-4, n=18 Day 5, n=16 Day 6, n=14 Day 7). Left graphs show the mean with standard deviation. Right graphs show the Box and Whiskers plots (line: median, Box is 25% and 75%, Ears: Minimum and Maximum). Analyzed using nonparametric two-way ANOVA (F1-LD-F1 design). ***** $p<0.05$ Sham vs SAH at the indicated time-point.

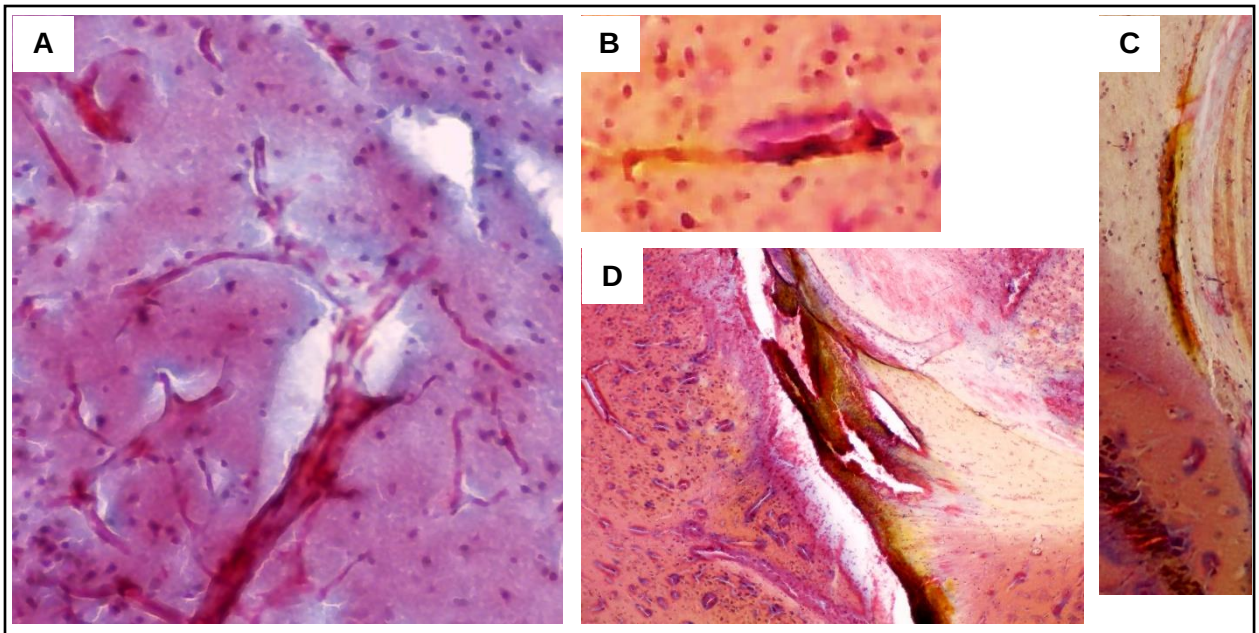


Figure IV. Representative Images of MSB staining. Red stains fibrin(ogen) (*e.g.* thrombi). Yellow stains erythrocytes and can stain new fibrin(ogen). Blue stains collagen. Cell nuclei are stained black. **A.** Several different microthrombi sizes can easily be identified. Thrombi (red) is easily differentiated from erythrocytes within blood vessels (**B**) and/or extravasated blood (**C**-**D**) (yellow). Poor/incomplete perfusion or lack of perfusion are possible reasons for erythrocytes to remain in blood vessels. Another possibility is erythrocytes were either trapped within a thrombus or trapped within the blood vessel immediately preceding a thrombi (as seen in **B**).

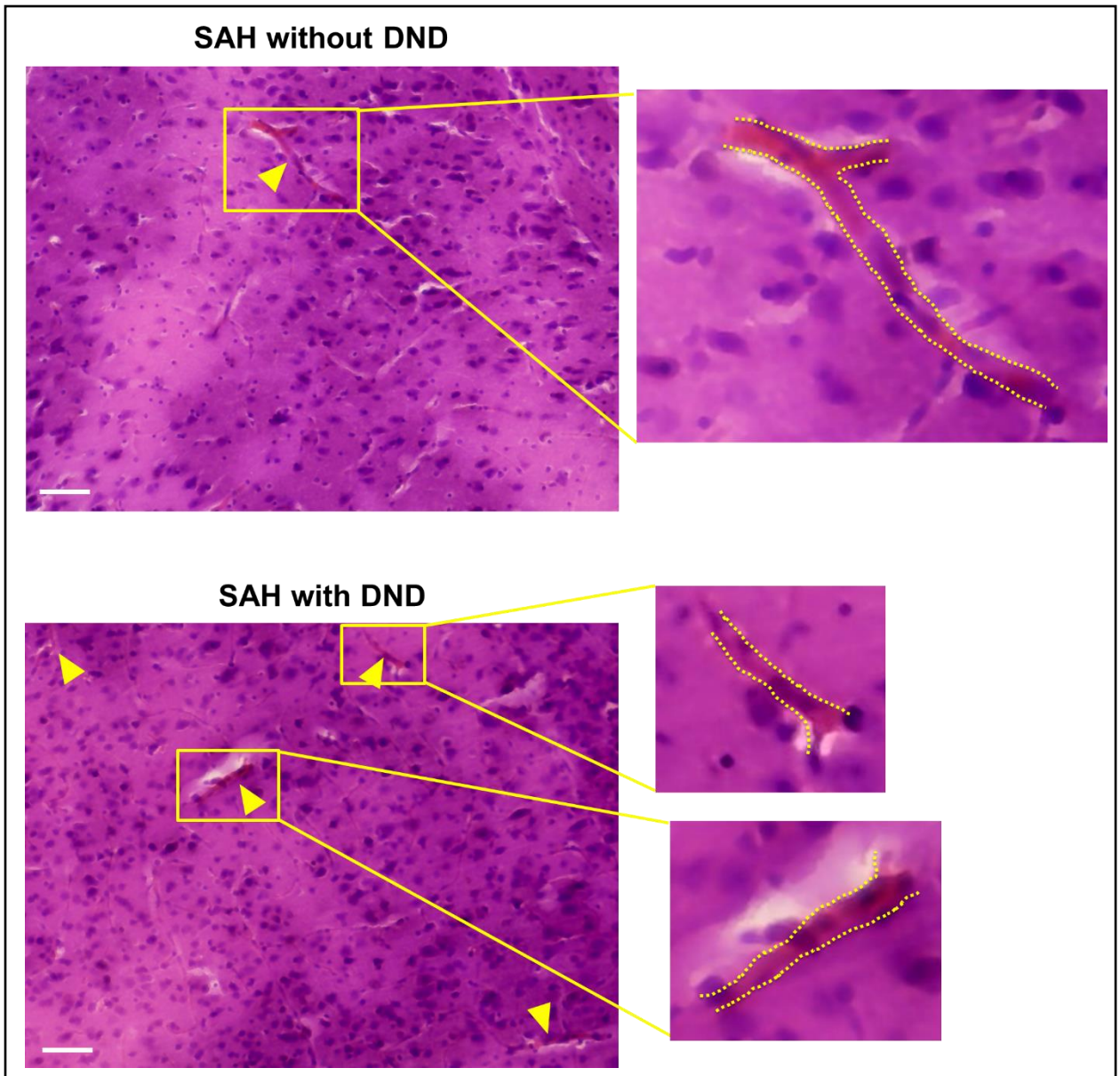


Figure V. Representative Images of Microthrombi in H&E staining. Thrombi are outlined and highlighted with arrowheads.

Table IV. Individual Mouse Performances on Neurobehavior and Death/Euthanasia Date from the 30 Males Experiencing SAH. Overall, 6/26 males (23%) surviving for more than 2 days had delayed neurological deficits. Day euthanized is the day the mouse was sacrificed for tissue collection. All mice with a value in the "Day of Histological Analysis" were used to assess vasospasm and microthrombi at the indicated day; mice with (DiI) were also used for vasospasm analysis via vessel painting. For SAH mice which did not develop DND, vasospasm and microthrombi were assessed on day 7. For SAH mice developed DND, vasospasm and microthrombi were assessed on day 5.7 (SD=1.37). Grey highlight shows mice which exhibited DND. Bold font indicates the day of delayed deficits. Mice 3, 26, and 28 died overnight before behavioral performance could be assessed on day 1. Mouse 19 died immediately following perforation despite resuscitation attempts. Mice 3, 19, 26 and 28 were excluded from analysis in this study.

	Neuroscore							Day of	Day of Histological
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Death/Euthanasia	Analysis
1	18	23	24	24	23	24	24	7	7
2	17	22	19	24	24	24	24	7	7
3								0	
4	21	23	20	24	24	24	24	7	
5	20	24	24	22	**19**	24	24	7	7 (DiI)
6	13	9	7					3	
7	19	22	22	23	24	24	24	7	7 (DiI)
8	10	16	**9**					3	3
9	17	23	24	23	24	24	24	7	
10	18	24	24	24	23	24	24	7	
11	17	19	21	17	**16**	24	24	7	7 (DiI)
12	17	17	23	21	23	24	24	7	7 (DiI)
13	12	13	14	12	**9**			5	5 (DiI)
14	20	23	24	21	24	24	24	7	
15	14	10	10	13	11	13	12	7	7
16	11	10	17	18	19	20	19	7	7
17	14	17	19	19	19	20	21	7	
18	12	11	15	16	18	18	21	7	7 (DiI)
19								0	
20	19	22	24	23	24	24	24	7	
21	13	21	18	19	19	20	21	7	
22	17	18	20	23	22	23	24	7	7 (DiI)
23	8	7						2	
24	17	21	24	24	24	24	24	7	7
25	20	21	20	23	21	**3**		6	6 (DiI)
26								0	
27	19	22	22	24	24	24	24	7	
28								0	
29	13	16	16	17	13	**8**		6	6
30	19	22	23	23	24	24	23	7	7 (DiI)

Table V. Individual Mouse Performances on Neurobehavior and Death/Euthanasia Date from the 20 Females Experiencing SAH. Overall, 9/19 females (47%) surviving for more than 2 days had delayed neurological deficits. Day euthanized is the day the mouse was sacrificed for tissue collection. All mice with a value in the "Day of Histological Analysis" were used to assess vasospasm and microthrombi at the indicated day; mice with (DiI) were also used for vasospasm analysis via vessel painting. For SAH mice which did not develop DND, vasospasm and microthrombi were assessed on day 7. For SAH mice developed DND, vasospasm and microthrombi were assessed on day 6 (SD=1.05). Grey highlight shows mice which exhibited DND. Bold font indicates the day of delayed deficits. Mouse 3 died 6 hours after SAH and was excluded from analysis in this study.

	Neuroscore							Day of Death/Euthanasia	Day of Histological Analysis
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7		
1	20	23	24	24	**19**	24	24	7	7 (DiI)
2	18	24	23	23	24	24	24	7	7
3								0	
4	17	20	**13**	24	24	**2**		6	6
5	18	22	24	23	24	24	24	7	7
6	13	15	16	**6**				4	4 (DiI)
7	13	19	**14**	23	**12**	6		6	6 (DiI)
8	17	23	23	23	**17**	23	22	7	7 (DiI)
9	19	23	23	24	24	24	24	7	7 (DiI)
10	20	22	23	23	23	24	23	7	7 (DiI)
11	20	22	**17**	15	12			5	5 (DiI)
12	20	21	23	24	**16**	16	15	7	7
13	18	20	22	24	24	24	23	7	7
14	17	23	20	23	23	24	20	7	7 (DiI)
15	21	20	21	23	**8**			5	5
16	17	23	23	24	**18**	20	23	7	7
17	15	20	22	23	23	23	23	7	7
18	13	20	23	20	22	23	24	7	7
19	15	16	14	21	22	23	23	7	7 (DiI)
20	17	18	19	23	22	24	23	7	7 (DiI)

Table VI. Microthrombi Statistical Analysis for Each Experimental Group for Each Sex. Sham n=8/sex, SAH without DND n=10/sex, SAH with DND n=6 males n=9 females. Microthrombi count is number per brain slice. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)	p-Value
MSB Staining		
Male		
Sham	3.375 (-0.1983 to 6.948)	N/A
SAH without DND	109.5 (71.15 to 149.9)	**0.0469** vs Sham
SAH with DND	491.2 (276.2 to 706.1)	**<0.0001** vs Sham **<0.0001** vs SAH without DND
Female		
Sham	3.0 (0.4721 to 5.528)	N/A
SAH without DND	94.90 (58.15 to 131.7)	**0.0485** vs Sham
SAH with DND	388.0 (268.2 to 507.8)	**<0.0001** vs Sham **0.0276** vs SAH without DND
H&E Staining		
Male		
Sham	2.625 (0.6803 to 4.570)	N/A
SAH without DND	42.40 (34.86 to 49.94)	**0.0219** vs Sham
SAH with DND	173.7 (62.17 to 285.2)	**<0.0001** vs Sham 0.0853 vs SAH without DND
Female		
Sham	2.250 (0.3673 to 4.133)	N/A
SAH without DND	59.20 (34.02 to 84.38)	**0.0405** vs Sham
SAH with DND	220.8 (130.2 to 311.3)	**<0.0001** vs Sham **0.0451** vs SAH without DND

Table VII. Microthrombi Statistical Analysis between Male and Female Mice. Sham n=8/sex, SAH without DND n=10/sex, SAH with DND n=6 males n=9 females. Microthrombi count is number per brain slice. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)	p-Value
MSB Staining		
Sham		
Male	3.375 (-0.1983 to 6.948)	N/A
Female	3.0 (0.4721 to 5.528)	0.8424 vs Male
SAH without DND		
Male	109.5 (71.15 to 149.9)	N/A
Female	94.90 (58.15 to 131.7)	0.5419 vs Male
SAH with DND		
Male	491.2 (276.2 to 706.1)	N/A
Female	388.0 (268.2 to 507.8)	0.2870 vs Male
H&E Staining		
Sham		
Male	2.625 (0.6803 to 4.570)	N/A
Female	2.250 (0.3673 to 4.133)	0.7481 vs Male
SAH without DND		
Male	42.40 (34.86 to 49.94)	N/A
Female	59.20 (34.02 to 84.38)	0.1655 vs Male
SAH with DND		
Male	173.7 (62.17 to 285.2)	N/A
Female	220.8 (130.2 to 311.3)	0.4451 vs Male

Table VIII. Microthrombi Statistical Analysis between Groups when Male and Female Data is Combined. Microthrombi count is number per brain slice. Sham n=8/sex, SAH without DND n=10/sex, SAH with DND n=6 males n=9 females. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)	p-Value
MSB Staining		
Sham	3.188 (1.279 to 5.096)	N/A
SAH without DND	102.2 (78.03 to 126.4)	**0.0148** vs Sham
SAH with DND	429.3 (330.8 to 527.7)	**<0.0001** vs Sham **<0.0001** vs SAH without DND
H&E Staining		
Sham	2.438 (1.254 to 3.621)	N/A
SAH without DND	50.80 (38.29 to 61.31)	0.0658 vs Sham
SAH with DND	201.9 (139.9 to 263.9)	**<0.0001** vs Sham **<0.0001** vs SAH without DND

Table IX. Correlations Between DND and Microthrombi, Vasospasm, or Infarction. The point-biserial correlation coefficients were computed to test for correlations between DND and microthrombi count, or large artery diameters, or infarct volume in SAH mice. Bold font denotes statistical significance.

	Correlation Coefficient, r (95% Confidence Interval)	p-Value
Microthrombi		
Male	0.8353 (0.5806 to 0.9415)	**<0.0001**
Female	0.8069 (0.5567 to 0.9229)	**<0.0001**
Large Artery Diameter		
L-ACA		
Male	-0.4450 (-0.8562 to 0.3112)	0.2300
Female	0.1582 (-0.5237 to 0.7166)	0.6624
R-ACA		
Male	-0.4325 (-0.8520 to 0.3252)	0.2450
Female	-0.3511 (-0.8032 to 0.3578)	0.3199
L-MCA		
Male	-0.06169 (-0.6973 to 0.6283)	0.8747
Female	-0.2658 (-0.7671 to 0.4371)	0.4579
R-MCA		
Male	0.1652 (-0.5606 to 0.7474)	0.6710
Female	-0.0064 (-0.6336 to 0.6259)	0.9860
L-ICA		
Male	-0.1984 (-0.7622 to 0.5366)	0.6089
Female	-0.5198 (-0.8661 to 0.1634)	0.1235
R-ICA		
Male	-0.5327 (-0.8841 to 0.2035)	0.1398
Female	-0.3774 (-0.8138 to 0.3310)	0.2823
BA		
Male	0.0334 (-0.6452 to 0.6825)	0.9321
	-0.4977 (-0.8584 to 0.1923)	0.1432
Infarct Volume		
Male	0.7562 (0.4031 to 0.9133)	**0.0017**
Female	0.6351 (0.2405 to 0.8495)	**0.0035**

Table X. Infarct Volume Statistical Analyses. Sham n=8/sex, SAH without DND n=10/sex, SAH with DND n=6 males n=9 females. Infarct areas were calculated every mm from +2 to -3 from Bregma in MSB stained slices and summed to obtain the volumes. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)	p-Value	
		Group Comparison	Male vs Female
Male			
Sham	0.43 (0.120 to 0.740)	N/A	N/A
SAH without DND	3.90 (2.47 to 5.34)	0.0754 vs Sham	N/A
SAH with DND	10.4 (4.25 to 16.6)	**<0.0001** vs Sham **0.0018** vs SAH without DND	N/A
Female			
Sham	0.41 (0.261 to 0.564)	N/A	0.9062 vs Male Sham
SAH without DND	3.12 (1.69 to 4.55)	0.6402 vs Sham	0.3957 vs Male SAH without DND
SAH with DND	11.3 (3.10 to 19.5)	**0.0043** vs Sham **0.0240** vs SAH without DND	0.8546 vs Male SAH with DND
Data Combined			
Sham	0.42 (0.271 to 0.572)	N/A	N/A
SAH without DND	3.51 (2.58 to 4.45)	0.1583 vs Sham	N/A
SAH with DND	11.0 (6.07 to 15.8)	**<0.0001** vs Sham **0.0002** vs SAH without DND	N/A

Table XI. Correlations Between Infarction and Microthrombi, Vasospasm, or Sex. The point-biserial correlation coefficients were computed to test for correlations between infarct volumes and microthrombi count, or large artery diameters, or sex in SAH mice. Bold font denotes statistical significance.

	Correlation Coefficient, r (95% Confidence Interval)	p-Value
Microthrombi		
Male	0.8353 (0.5806 to 0.9415)	**<0.0001**
Female	0.8069 (0.5567 to 0.9229)	**<0.0001**
Large Artery Diameter		
L-ACA		
Male	-0.4450 (-0.8562 to 0.3112)	0.2300
Female	0.1582 (-0.5237 to 0.7166)	0.6624
R-ACA		
Male	-0.4325 (-0.8520 to 0.3252)	0.2450
Female	-0.3511 (-0.8032 to 0.3578)	0.3199
L-MCA		
Male	-0.06169 (-0.6973 to 0.6283)	0.8747
Female	-0.2658 (-0.7671 to 0.4371)	0.4579
R-MCA		
Male	0.1652 (-0.5606 to 0.7474)	0.6710
Female	-0.0064 (-0.6336 to 0.6259)	0.9860
L-ICA		
Male	-0.1984 (-0.7622 to 0.5366)	0.6089
Female	-0.5198 (-0.8661 to 0.1634)	0.1235
R-ICA		
Male	-0.5327 (-0.8841 to 0.2035)	0.1398
Female	-0.3774 (-0.8138 to 0.3310)	0.2823
BA		
Male	0.0334 (-0.6452 to 0.6825)	0.9321
	-0.4977 (-0.8584 to 0.1923)	0.1432
Infarct Volume		
Male	0.7562 (0.4031 to 0.9133)	**0.0017**
Female	0.6351 (0.2405 to 0.8495)	**0.0035**

Table XII. Large Artery Diameter Statistical Analysis for Male Mice. Diameters (μm) were determined using vessel painted (DiI) brains. Sham n=5, SAH without DND n=5, SAH with DND n=4. L: left, R: right, ACA: anterior cerebral artery, MCA: middle cerebral artery. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)	p-Value
L-ACA		
Sham	127.6 (122.7 to 132.5)	N/A
SAH without DND	12.8 (116.3 to 131.3)	0.8173 vs Sham
SAH with DND	113.3 (86.39 to 140.1)	0.1200 vs Sham 0.2869 vs SAH without DND
R-ACA		
Sham	12704 (122.0 to 132.8)	N/A
SAH without DND	110.8 (92.59 to 129.0)	0.0689 vs Sham
SAH with DND	100.0 (85.02 to 115.0)	**0.0063** vs Sham 0.3109 vs SAH without DND
L-MCA		
Sham	128.4 (120.3 to 136.5)	N/A
SAH without DND	112.4 (91.41 to 133.4)	0.0932 vs Sham
SAH with DND	110.5 (82.08 to 138.9)	0.1133 vs Sham >0.9999 vs SAH without DND
R-MCA		
Sham	124.8 (119.4 to 130.2)	N/A
SAH without DND	104.0 (87.76 to 120.2)	**0.0122** vs Sham
SAH with DND	107.3 (95.90 to 118.6)	**0.0300** vs Sham 0.6052 vs SAH without DND
L-ICA		
Sham	147.2 (141.8 to 152.6)	N/A
SAH without DND	135.8 (122.3 to 149.3)	0.1118 vs Sham
SAH with DND	129.8 (93.99 to 165.5)	0.1228 vs Sham >0.9999 vs SAH without DND
R-ICA		
Sham	153.0 (142.0 to 164.0)	N/A
SAH without DND	150.2 (136.0 to 164.4)	0.9216 vs Sham
SAH with DND	136.0 (113.4 to 158.6)	0.1131 vs Sham 0.2000 vs SAH without DND
BA		
Sham	172.4 (154.9 to 189.9)	N/A
SAH without DND	166.2 (152.9 to 179.5)	0.6697 vs Sham
SAH with DND	166.8 (155.7 to 177.8)	0.7416 vs Sham 0.9971 vs SAH without DND

Table XIII. Large Artery Diameter Statistical Analysis for Female Mice. Diameters (μm) were determined using vessel painted (DiI) brains. Sham n=5, SAH without DND n=5, SAH with DND n=5. L: left, R: right, ACA: anterior cerebral artery, MCA: middle cerebral artery. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)	p-Value
L-ACA		
Sham	122.2 (114.8 to 129.6)	N/A
SAH without DND	96.20 (57.47 to 134.9)	0.1604 vs Sham
SAH with DND	103.4 (82.30 to 124.5)	0.3575 vs Sham 0.8497 vs SAH without DND
R-ACA		
Sham	119.8 (112.6 to 127.0)	N/A
SAH without DND	106.6 (96.49 to 116.7)	0.4067 vs Sham
SAH with DND	94.00 (62.60 to 125.4)	0.0565 vs Sham 0.4381 vs SAH without DND
L-MCA		
Sham	122.0 (112.0 to 132.0)	N/A
SAH without DND	109.4 (92.29 to 126.5)	0.3624 vs Sham
SAH with DND	101.4 (78.63 to 124.2)	0.0912 vs Sham 0.6498 vs SAH without DND
R-MCA		
Sham	127.6 (121.5 to 133.7)	N/A
SAH without DND	100.2 (90.93 to 109.5)	**0.0292** vs Sham
SAH with DND	100.0 (70.66 to 129.3)	**0.0281** vs Sham 0.9997 vs SAH without DND
L-ICA		
Sham	135.2 (123.5 to 146.9)	N/A
SAH without DND	119.4 (102.3 to 136.5)	0.2890 vs Sham
SAH with DND	100.6 (75.57 to 125.6)	**0.0156** vs Sham 0.7726 vs SAH without DND
R-ICA		
Sham	140.0 (133.0 to 147.0)	N/A
SAH without DND	129.6 (105.8 to 153.4)	0.9145 vs Sham
SAH with DND	113.6 (83.27 to 143.9)	**0.0398** vs Sham 0.4406 vs SAH without DND
BA		
Sham	158.6 (150.4 to 166.8)	N/A
SAH without DND	157.8 (140.0 to 175.6)	0.9912 vs Sham
SAH with DND	146.2 (137.4 to 155.0)	0.1635 vs Sham 0.1992 vs SAH without DND

Table XIV. Large Artery Diameter Statistical Analysis between Male and Female Mice. Diameters (μm) were determined using vessel painted (DiI) brains. Sham n=5/sex, SAH without DND n=5/sex, SAH with DND n=4 males n=5 females. L: left, R: right, ACA: anterior cerebral artery, MCA: middle cerebral artery. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)		p-Value for
	Male	Female	Male vs Female
L-ACA			
Sham	127.6 (122.7 to 132.5)	122.2 (114.8 to 129.6)	0.1310
SAH without DND	12.8 (116.3 to 131.3)	96.20 (57.47 to 134.9)	0.0880
SAH with DND	113.3 (86.39 to 140.1)	103.4 (82.30 to 124.5)	0.4149
R-ACA			
Sham	12704 (122.0 to 132.8)	119.8 (112.6 to 127.0)	**0.0470**
SAH without DND	110.8 (92.59 to 129.0)	106.6 (96.49 to 116.7)	0.5910
SAH with DND	100.0 (85.02 to 115.0)	94.00 (62.60 to 125.4)	0.6695
L-MCA			
Sham	128.4 (120.3 to 136.5)	122.0 (112.0 to 132.0)	0.2045
SAH without DND	112.4 (91.41 to 133.4)	109.4 (92.29 to 126.5)	0.7662
SAH with DND	110.5 (82.08 to 138.9)	101.4 (78.63 to 124.2)	0.4788
R-MCA			
Sham	124.8 (119.4 to 130.2)	127.6 (121.5 to 133.7)	0.3650
SAH without DND	104.0 (87.76 to 120.2)	100.2 (90.93 to 109.5)	0.5880
SAH with DND	107.3 (95.90 to 118.6)	100.0 (70.66 to 129.3)	0.5767
L-ICA			
Sham	147.2 (141.8 to 152.6)	135.2 (123.5 to 146.9)	**0.0325**
SAH without DND	135.8 (122.3 to 149.3)	119.4 (102.3 to 136.5)	0.0701
SAH with DND	129.8 (93.99 to 165.5)	100.6 (75.57 to 125.6)	0.0793
R-ICA			
Sham	153.0 (142.0 to 164.0)	140.0 (133.0 to 147.0)	**0.0242**
SAH without DND	150.2 (136.0 to 164.4)	129.6 (105.8 to 153.4)	0.0728
SAH with DND	136.0 (113.4 to 158.6)	113.6 (83.27 to 143.9)	0.1504
BA			
Sham	172.4 (154.9 to 189.9)	158.6 (150.4 to 166.8)	0.0826
SAH without DND	166.2 (152.9 to 179.5)	157.8 (140.0 to 175.6)	0.3247
SAH with DND	166.8 (155.7 to 177.8)	146.2 (137.4 to 155.0)	**0.0033**

Table XV. Large Artery Diameter Statistical Analysis between Groups when Male and Female Data is Combined. Diameters (μm) were determined using vessel painted (DiI) brains. Sham n=5/sex, SAH without DND n=5/sex, SAH with DND n=4 males n=5 females. L: left, R: right, ACA: anterior cerebral artery, MCA: middle cerebral artery. Bold font denotes statistical significance.

	Mean (95% Confidence Interval)	p-Value
L-ACA		
Sham	124.9 (120.9 to 128.9)	N/A
SAH without DND	110.0 (91.62 to 128.4)	0.1740 vs Sham
SAH with DND	107.8 (94.96 to 120.6)	0.1164 vs Sham 0.9611 vs SAH without DND
R-ACA		
Sham	123.6 (119.1 to 128.1)	N/A
SAH without DND	108.7 (100.5 to 116.9)	**0.0432** vs Sham
SAH with DND	96.67 (82.02 to 111.3)	**0.0004** vs Sham 0.1306 vs SAH without DND
L-MCA		
Sham	125.2 (119.7 to 130.7)	N/A
SAH without DND	110.9 (100.4 to 121.4)	0.0579 vs Sham
SAH with DND	105.4 (91.89 to 119.0)	**0.0140** vs Sham >0.9999 vs SAH without DND
R-MCA		
Sham	126.2 (122.9 to 129.5)	N/A
SAH without DND	102.1 (94.78 to 109.4)	**0.0003** vs Sham
SAH with DND	103.2 (89.62 to 116.8)	**0.0005** vs Sham 0.8380 vs SAH without DND
L-ICA		
Sham	141.2 (124.5 to 147.9)	N/A
SAH without DND	127.6 (117.2 to 138.0)	0.1171 vs Sham
SAH with DND	113.6 (94.28 to 132.8)	**0.0119** vs Sham >0.9999 vs SAH without DND
R-ICA		
Sham	146.5 (139.5 to 153.5)	N/A
SAH without DND	139.9 (126.7 to 153.1)	0.6819 vs Sham
SAH with DND	123.6 (106.1 to 141.0)	**0.0226** vs Sham 0.1262 vs SAH without DND
BA		
Sham	165.5 (156.4 to 174.6)	N/A
SAH without DND	162.0 (152.9 to 171.1)	0.8122 vs Sham
SAH with DND	155.3 (145.6 to 165.1)	0.2083 vs Sham 0.4966 vs SAH without DND

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*** 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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