Subarachnoid Hemorrhage Pattern Predicts Acute Cerebral Blood Flow Response in the Rat

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Abstract There is considerable variability in the presentation of patients with acute subarachnoid hemorrhage (aSAH). Evidence suggests that a thick, diffuse clot better predicts the development of delayed cerebral ischemia and poor outcomes. In a rodent model of acute SAH, we directly measured the effects of the volume of blood injected versus the pattern of distribution of hemorrhage in the subarachnoid space on markers of early brain injury, namely, cerebral blood flow (CBF), cerebrospinal fluid (CSF) concentrations of P450 eicosanoids and catecholamines, and cortical spreading depolarizations (CSDs). There is a significant decrease in CBF, an increase in CSF biomarkers, and a trend toward increasing frequency and severity of CSDs when grouped by severity of hemorrhage but not by volume of blood injected. In severe hemorrhage grade animals, there was a progressive decrease in CBF after successive CSD events. These results suggest that the pattern of SAH (thick diffuse clots) correlates with the "clinical" severity of SAH.

Keywords Subarachnoid hemorrhage · P450 eicosanoids · 20-HETE · 14,15-EET · Cortical spreading depolarization · Early brain injury · Stroke glymphatic

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Introduction

Aneurysmal subarachnoid hemorrhage (aSAH) is a devastating form of stroke with an incidence of approximately 9 in 100,000 person-years [1]. This condition results in immediate mortality of approximately 22%. There is a nearly 50% mortality rate in the first month, and of those patients surviving at least 1 year, 46–60% remain dependent [2]. One major hallmark of aSAH is the marked variability in clinical presentation, radiographic appearance, and long-term outcomes across patients. Furthermore, many patients, but not all, develop delayed cerebral ischemia (DCI) that, in the past, has been attributed to large vessel vasospasm due to numerous vasoactive compounds in the subarachnoid space. Progressive iterations of radiographic grading scales have improved the prediction of DCI by accounting for diffuse thick hemorrhage [3–5]. In addition, several recent reports have shown that the *pattern* of SAH and not the *volume* of blood per se, in patients with *non*-aneurysmal SAH is correlated with the development of angiographic vasospasm and delayed infarcts [6–8].

Early brain injury (EBI), damage sustained within the first 72 h after ictus, leads to delayed pathological changes such as brain tissue hypoxia, cerebral inflammation, and blood-brain barrier disruption [9, 10]. EBI plays a primary role in the initial clinical presentation of patients with SAH and contributes to secondary injuries, including DCI and subsequent poor prognosis [10]. Consequently, multiple groups have been studying the effects of various biomarkers after aSAH. The P450 eicosanoids are vasoactive and inflammatory products of arachidonic acid metabolism that have been shown to be present in the spinal fluid of aSAH patients. These metabolites play numerous and often opposing roles. Some have been shown to be vasoconstrictive and contribute to DCI [11, 12], and others have been shown to be neuroprotective in cerebral ischemia/reperfusion injury and can reduce cerebral edema [13, 14].

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Epoxyeicosatrienoic acids (EETs) are vasodilatory, anti-inflammatory, and antithrombotic [15]. 14,15-EET may protect against the development of DCI in both human and animal studies [16, 17], whereas 20-hydroxyeicoast-etraenoic acid (20-HETE) and 5-hydroxytryptamine (5-HT) are associated with early and delayed vasospasm [11, 12]. Another hallmark brain injury after SAH is the presence of cortical spreading depolarizations (CSDs) that may contribute to the development of DCI [18]. These are self-propagating waves of neuronal and glial depolarization that trigger a vascular response through neurovascular coupling. The vascular response can range from a spreading hyperemia to a pronounced oligemia and can lead to significant ischemia and cell death [19]. Under normal conditions, CSDs lead to increased cerebral metabolic demand and trigger a hemodynamic response composed of multiple opposing vasomotor influences at various stages of the CSD wave [20]. In the pathologic state after SAH CSDs lead to paradoxical hypoperfusion during the period of elevated metabolic demand which leads to tissue damage [21]. CSDs have been well-documented in animal models and human patients with severe SAH [19, 21]. We propose that a thick diffuse pattern of hemorrhage is the driver of the cascade of events that occurs after hemorrhage rather than a dose response to the spasmogens in the extravasated blood.

In this study, we directly compare the *pattern* of blood in experimental SAH [22, 23] to the *volume* of blood on cerebral blood flow, the CSF expression of P450 eicosanoids, and the development of CSDs. We find that a thick diffuse hemorrhage most closely correlates with the severity of injury after SAH.

Materials and Methods

Animals and Surgical Preparation

This study protocol was approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University, which is in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All institutional and national guidelines for the care and use of laboratory animals were followed. As previously published [22], rats were anesthetized with isoflurane, catheterized (central venous and arterial), placed in a stereotactic frame, and maintained in a “lightly anesthetized state” with i.v. Brevital. The skull was thinned for laser Doppler flowmetry and optical intrinsic signaling (OIS) and a craniotomy performed for placement of a spinal needle in the prechiasmatic cistern for injection of autologous blood into the subarachnoid space.

Experimental Subarachnoid Hemorrhage and Recording

Freshly drawn autologous blood of varying volumes (50 μL, 100 μL, 200 μL, and 250 μL) was infused over 1 min through the spinal needle to minimize changes in intracranial pressure (ICP) [24, 25]. Control animals received an injection of 200 μL of artificial cerebral spinal fluid (aCSF). CBF, mean arterial pressure (MAP), flick latency, and temperature were monitored during a 10-min baseline to ensure a stable anesthetic plane and for an additional 30 min after induction of SAH. OIS was recorded using a multispectral imaging system using QiOptiq Optem FUSION (QiOptiq, NY) modular lens system. A broadband light from a fiber-optic illuminator filtered through a liquid crystal tunable filter (VariSpec VIS, Perkin-Elmer, MA) was used to illuminate the thin skull preparation 3 mm right lateral to the sagittal suture and 2 mm posterior to the coronal suture, at 30° angle with respect to the optical axis of the imaging system. The reflectance from the sample was acquired by monochrome camera (Model: Flea2 IEEEb, Point Grey Research, Inc., Canada) controlled by custom software written using LabVIEW (National Instruments, Austin, TX).

Cerebrospinal Fluid Collection

The rat was given a bolus of 0.5 mL sodium Brevital (20 mg/mL), and the head angled ventrally 30°. A 27-gauge needle was percutaneously inserted into the cisterna magna and 100–200 μL of CSF aspirated. CSF was centrifuged for 10 s at 10,000 RPM and then at −80 °C. Control CSF was obtained from anesthetized naïve rats in a similar fashion. All samples were analyzed for P450 eicosanoids and catecholamines by the lipidomics core on a mass spectrometer. Following CSF collection, the animals were euthanized by sodium Brevital overdose and perfusion fixed with 100 mL saline followed by 100 mL 10% formalin. The whole brain was removed carefully and images of the ventral and dorsal surfaces obtained. The hemorrhage grade was assigned as previously described [22]. Briefly, the ventral surface of the brain was partitioned into six sections. Each section was allotted a number from 0 to 3 depending on the amount of subarachnoid blood in the section: 0 no blood, 1 minimal blood, 2 moderate blood clot with recognizable arteries, and 3 blood clot obliterating all arteries. Scores were summed from all six sections. Scores were categorized as follows: 0–7 mild, 8–12 moderate, and 13–18 severe.

Analysis

CBF was normalized to the baseline. The mean percent change, in 5-min epochs, was calculated for each group and compared using the one-way ANOVA and post hoc Tukey test. Differences in the mean concentrations of metabolite markers in the CSF were compared using the one-way ANOVA and post hoc Tukey test. The number of cortical spreading depressions was compared with the nonparametric Kruskal-Wallis test. Statistics were performed with MATLAB and IBM SPSS v24 software.

Results

There were a total of 31 experimental animals. Five (5) animals were given a 250 μL injection, eight (8) animals were given a 200 μL injection, four (4) animals were given a 100 μL injection, and four (4) animals were given a 50 μL injection. Ten (10) animals were given an aCSF injection. This resulted in fourteen (14) mild hemorrhages, eight (8) moderate hemorrhages, and nine (9) severe hemorrhages. There were no severe hemorrhages in the 50 μL group (2/4 mild and 2/4 moderate). Of the other three injection groups, there were 25% (1/4), 62.5% (5/8), and 60% (3/5) severe hemorrhages in the 100 μL, 200 μL, and 250 μL injection groups, respectively.

Cerebral Blood Flow

CBF was reduced immediately after experimental SAH in all animals; however, there was a large amount of variability in the severity of the reduction. CBF categorized according to the volume of blood injected was only significantly reduced from aCSF injection in the 250 μL group (the largest volume injected) due to the large variability in responses across groups (Fig. 1a). By contrast, there is a significant difference in relative CBF depending on the hemorrhage grade of the animal (Fig. 1b) consistent with previous reports [22]. There is a substantial decrease in CBF in the early phase after SAH injection compared to the baseline in the moderate and severe groups. The differences between mild, moderate, and severe hemorrhages are significant for the first minutes after injection ($P < 0.05$), after which differences between mild and severe hemorrhages persist for 20 min post-injection ($P < 0.05$).

Cerebrospinal Fluid

The concentration of 14,15-EET was significantly elevated in all hemorrhage grades compared to naïve controls ($P < 0.01$). When grouped by injected blood volume, only the largest blood volume group was statistically different ($P < 0.05$) likely due to the large variability. Similarly, the mean concentrations of 20-HETE are significantly different when grouped by hemorrhage severity but not by volume injected. The mean concentration of 5-HT is significantly different in severe hemorrhage compared to control ($P < 0.01$) and moderate hemorrhage ($P < 0.05$), whereas there is no significance when grouped by volume injected. There was no significant difference in the CSF concentration of norepinephrine (NE) independent of grouping (Fig. 2 and Table 1).

Cortical Spreading Depolarization

The number of CSD events were compared between hemorrhage grades as well as volume of blood injected. Of the 14 animals with mild hemorrhages, 3 experienced CSD events; of the 8 moderate hemorrhage animals, 3 experienced CSD events; and of the 9 severe hemorrhage animals, 5 experienced CSD events. There was a trend toward increased frequency of CSD events in severe hemorrhages, but it did not quite reach significance ($P = 0.064$). In animals with multiple CSD events, subsequent CSD events appeared different between mild and severe hemorrhage grades (Fig. 3). In the mild hemorrhage animal, the initial CSD event was a brief period of oligemia followed by hyperemia. In subsequent CSD events, oligemia diminished or was absent and the hyperemic response increased. In the moderate hemorrhage animal, there is a more gradual shift from oligemia to hyperemia. In the severe hemorrhage animal, successive CSD events lead to progressively worsening oligemia and a loss of the hyperemic component (Fig. 3).

Discussion

In this study we examined the correlation between the volume of blood injected in the subarachnoid space and the distribution or pattern of SAH on markers of early brain injury: cerebral perfusion, CSF levels of the P450 eicosanoids and catecholamines (14,15-EET, 20-HETE, 5-HT, and NE), as well as the development of CSDs and their effects on CBF. We found that an acute decrease in cerebral perfusion was quite variable across animals if grouped by the volume of blood injected. By contrast, Sugawara grade correlated closely with the severity of cerebral perfusion deficits and levels of the vasoactive metabolites 20-HETE, 5-HT, and 14–15-EET. There was a trend toward increasing frequency of CSD events in high-grade hemorrhages, and these CSD profiles demonstrated worsening oligemia compared to those associated with low-grade hemorrhages. These changes

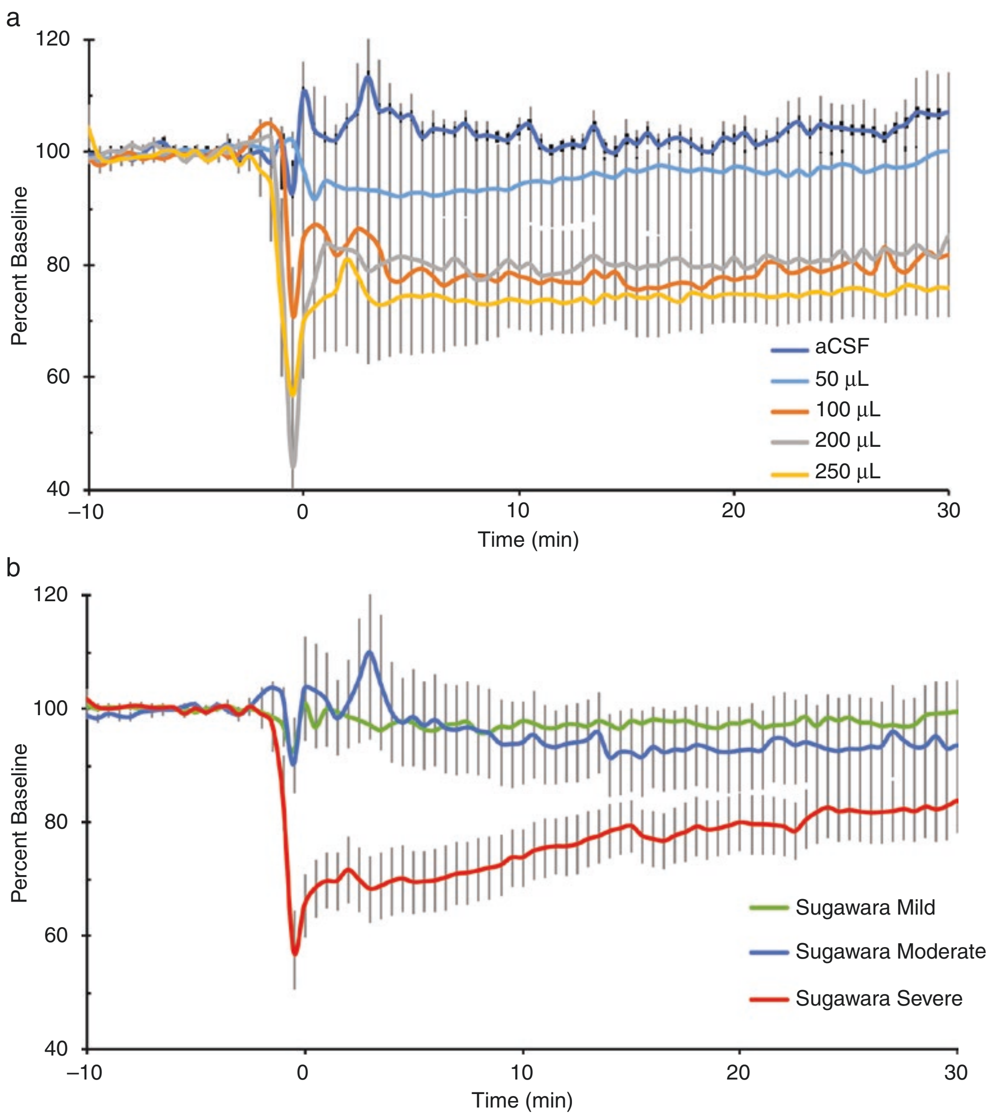

Fig. 1 rCBF after induced SAH (**a**) When grouped by volume of blood injected, only the 250 µL group demonstrated a significant difference from controls (aCSF injection) and was not different from other injected blood volume groups. (**b**) When grouped by pattern of clot in subarachnoid space (Sugawara grade), there is a substantial decrease in rCBF immediately after blood injection in the moderate and severe groups compared to the baseline. Mild, moderate, and severe hemorrhages are significantly different from each other until 5 min after injection ($P < 0.05$), after which mild and severe hemorrhages remain significantly different until 20 min after the injection ($P < 0.05$)

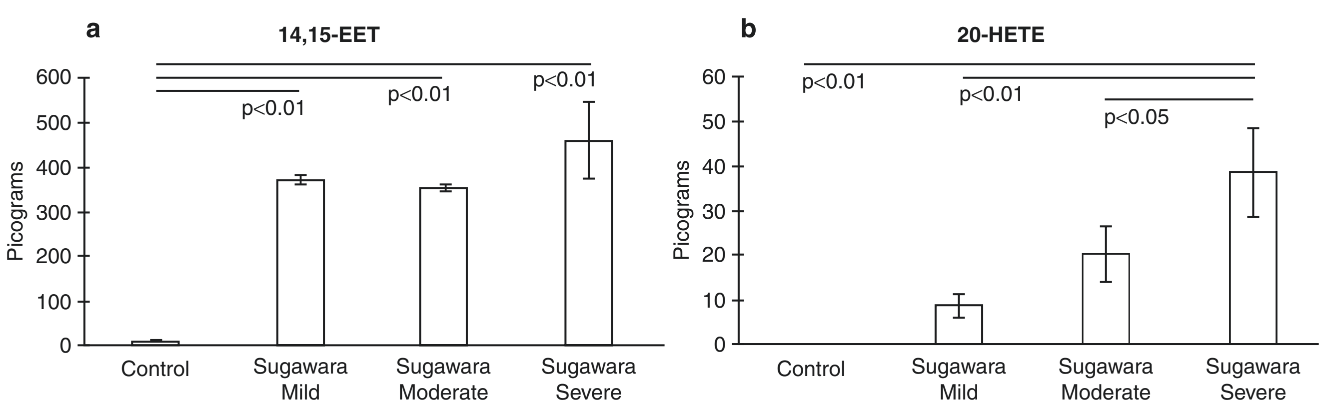

Fig. 2 (**a**) CSF concentrations of 14,15-EET increased in all hemorrhage grades after experimental hemorrhage. (**b**) CSF concentrations of 20-HETE increased proportionally with increasing severity of hemorrhage grade

Table 1 Eicosanoid concentrations by hemorrhage grade and volume injected blood

	Hemorrhage Grade				Injection Volume		
Metabolite	Control	Sugawara Mild	Sugawara Moderate	Sugawara Severe	aCSF	50&100 μL	200&250 μL
14,15-EET	6.75 ± 3.94	371.5 ± 11.44*	354.4 ± 6.66*	460.8 ± 87.5*	396.1 ± 45.77	440.7 ± 97.80	458.3 ± 121.65°
20-HETE	0	8.7 ± 2.52	20.2 ± 6.31	38.6 ± 10.20*#‡	1.5 ± 1.45	19.9 ± 7.55	22.1 ± 9.10
5-HT	0.09 ± 0.02	18 ± 5.70	11.6 ± 5.23	44.4 ± 10.91*#	13.1 ± 4.78	19.6 ± 8.34	28.0 ± 8.86
NE	0.58 ± 0.03	1.80 ± 1.27	3.43 ± 2.63	0.97 ± 0.21	3.24 ± 1.99	0.46 ± 0.23	0.53 ± 0.20

Units are pg/100 μL for the eicosanoids (EETs, HETEs, and DHETs) and units are ng/mL for NE
$^*P < 0.01$ when compared to Control; $^\circ P < 0.05$ when compared to Control; # $P < 0.05$ when compared to Moderate; ‡ $P < 0.01$ when compared to Mild

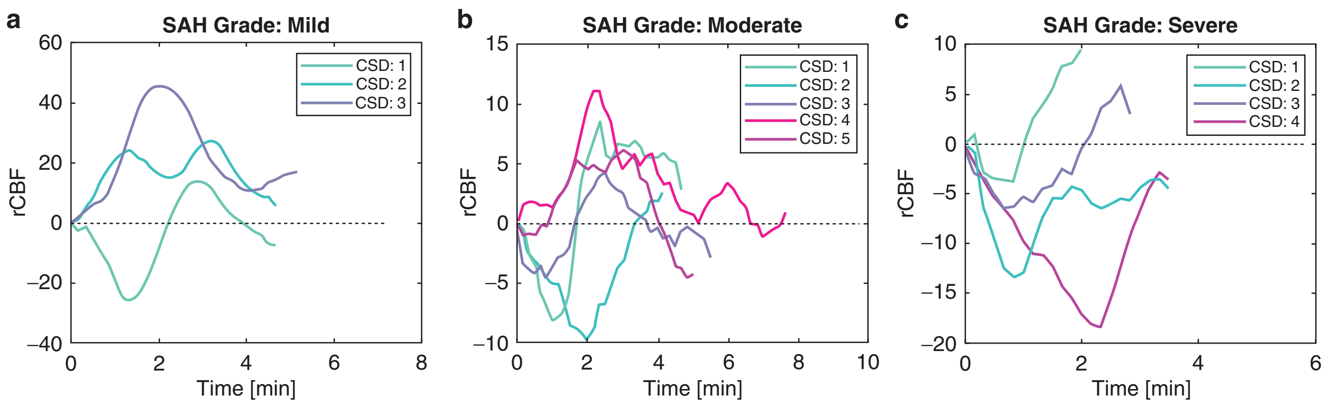

Fig. 3 rCBF changes in successive CSD events. (**a**) An animal with mild-grade hemorrhage and three spontaneous CSD events. Initial oligemia of the first CSD is absent in subsequent CSD events. (**b**) An animal with moderate-grade hemorrhage and five spontaneous CSD events. Initial oligemia persists for four CSD events but begins to diminish after the second event and is absent in the fifth event. The late hyperemic phase increases in magnitude with each successive wave. In high-grade hemorrhage, there is an initial oligemia with a mild compensatory hyperemia that progresses to severe, uncompensated oligemia. (**c**) An animal with severe-grade hemorrhage and four CSD events. Initial oligemia increases with each successive event, while the compensatory hyperemia diminishes and is eventually absent in the third and fourth waves

likely contribute to early brain injury and prime the brain for DCI [26] .

Acute global ischemia is well-known to accompany high clinical grade hemorrhages. Patients have poor neurological function, cerebral edema, and at times early infarcts [27, 28]. In our study, animals with large perfusion deficits had significant increases in the CSF concentration of 20-HETE, 14,15-EET, and 5-HT. Previous work has shown that an increase in 5-HT stimulates the synthesis and/or release of 20-HETE, which is a powerful vasoconstrictor [11] that contributes to vasospasm and poor outcomes in animal models and human studies [17, 29, 30]. 14,15-EET, on the other hand, is a potent vasodilator [17] and has been shown to be neuroprotective in ischemic stroke [31, 32], reduce perivascular inflammation [14], and decrease DCI [17, 33] after SAH. Different genetic polymorphisms of soluble epoxide hydrolase (sEH), an enzyme that metabolizes EETs into inactive secondary products, have been shown to correlate with neurologic outcomes after SAH [14, 16, 33]. Human polymorphisms with decreased sEH activity or animal models of knockout sEH demonstrated improved neurologic outcomes. Both 14,15-EET and 20-HETE are elevated in the CSF of patients with poor clinical grade [17, 30, 33]. Consistent with these clinical studies, we demonstrate elevated 20-HETE and 14,15-EET with severe hemorrhage grades. Interestingly, 14,15-EET is also elevated in low-grade hemorrhages suggesting that an increase in 14,15-EET is a compensatory response to SAH to maintain cerebral blood flow and that it is overwhelmed by the increased vasoconstrictive tone secondary to 20-HETE and 5-HT release in high-grade hemorrhages [17, 34].

Though previous work has reported elevated CSF epinephrine levels in human subjects at a higher risk for early death or disability [34], the CSF concentration of NE was not significantly different regardless of animals grouped by hemorrhage grade or volume of blood injected. The predictive value of elevated NE and epinephrine was weak with low sensitivity and specificity in the human study, and our finding is consistent given the small sample size. It has also been shown that plasma concentrations of catecholamines can be increased after SAH [35]. Our data did not demonstrate consistently elevated catecholamines in the CSF in high-grade hemorrhages, which suggests that catecholamine release associated with SAH is peripheral rather than central. Interestingly, we did not see a close relationship between CSF marker and volume of blood injected which would have been expected if CSF levels of metabolites were due to their presence in the peripheral blood used for SAH. In our model, ICP is controlled which may impact the degree of plasma catecholamines in the CSF sample due to a diminished Cushing's response.

Consistent with the findings of reduced CBF and increased CSF levels of 20-HETE, there is a trend toward increasing frequency of CSD events with increasing hemorrhage grade. Increased 20-HETE reduces CBF through vasoconstriction and has also been shown to be released at the level of the capillaries after CSD events [36] potentially exacerbating underlying ischemia. Interestingly, the CSD events in an animal with severe hemorrhage and multiple CSD events demonstrated increasing oligemia with each successive event (Fig. 3). By contrast, in animals with mild and moderate hemorrhages, the successive CSD events demonstrated hyperemic responses suggesting intact compensatory systems. Without these systems, animals with severe hemorrhages are at an increased risk of global ischemia and potential infarction due to decoupling of the neurovascular unit and an inability to compensate for the elevated metabolic demand [37].

The risk of DCI is also likely related to the diffuse distribution of blood through the subarachnoid space. Previous work has demonstrated the importance of a paravascular, glymphatic, waste clearance mechanism in the brain which is disrupted by blood in the subarachnoid space [38–40]. Tissue plasminogen activator (tPA) injected into the intraventricular space improves glymphatic perfusion and restores CSF flow [41–43] and cortical perfusion [43] after experimental SAH. In addition, parenchymal CSF flow is severely impaired secondary to perivascular blood clots which supports a localized effect of blood and its contribution to DCI [39]. Our model and data support the idea that the diffuse, thick distribution of subarachnoid blood impairs paravascular, glymphatic CSF flow and increases the incidence of EBI and DCI.

Conclusion

In conclusion, this study demonstrates no significant correlation between the volume of blood injected into the subarachnoid space and the severity of induced SAH as measured by the modified Sugawara grade. When grouped by hemorrhage grade, there is a significant decreased initial post SAH CBF; increased CSF concentrations of 20-HETE, 14,15-EET, and 5-HT; and a trend toward increased CSD events in high Sugawara grade experimental SAH. Our model confirms that the diffuse, thick pattern of SAH leads to increased early brain injury and therefore a likely increased risk of DCI.

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Conflict of Interest: **The authors declare that they have no conflict of interest.**

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