ORIGINAL ARTICLE

Relationship Between Hematoma Expansion Induced by Hypertension and Hyperglycemia and Blood–brain Barrier Disruption in Mice and Its Possible Mechanism: Role of Aquaporin-4 and Connexin43

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Abstract We aimed to select an optimized hematoma expansion (HE) model and investigate the possible mechanism of blood–brain barrier (BBB) damage in mice. The results showed that HE occurred in the group with hypertension combined with hyperglycemia (HH-HE) from 3 to 72 h after intracerebral hemorrhage; this was accompanied by neurological deficits and hardly influenced the survival rate. The receiver operating characteristic curve suggested the criterion for this model was hematoma volume expansion $\geq 45.0\%$. Meanwhile, HH-HE aggravated BBB disruption. A protector of the BBB reduced HH-HE, while a BBB disruptor induced a further HH-HE. Aquaporin-4 (AQP4) knock-out led to larger hematoma volume and more severe BBB disruption. Furthermore, hematoma volume and BBB disruption were reduced by

multiple connexin43 (Cx43) inhibitors in the wild-type group but not in the AQP4 knock-out group. In conclusion, the optimized HE model is induced by hypertension and hyperglycemia with the criterion of hematoma volume expanding $\geq 45.0\%$. HH-HE leads to BBB disruption, which is dependent on AQP4 and Cx43.

Keywords Intracerebral hemorrhage · Hematoma expansion · Animal model · Blood–brain barrier · Aquaporin-4 · Connexin43

Introduction

Intracerebral hemorrhage (ICH) is the second most common cause of cerebrovascular diseases and 75% of patients experience severe disability or even death [1]. Hematoma expansion (HE), which usually occurs at the early stage of ICH in 20%–40% of patients, independently predicts death and disability [2]. Although HE is predictable by radiological and laboratory findings [3, 4], its treatment is still limited. One of the crucial issues that limits the development of HE treatment is the lack of appropriate animal models, so that translational research is not feasible. The existing HE models are mainly induced by anticoagulants including warfarin, dabigatran, and rivaroxaban after creating an ICH model by collagenase injection [5–7]. However, collagenase dissolves the extracellular matrix and causes rupture of blood vessels, leading to gradual extravasation. Thus the hematoma itself expands within the first few hours, which differs from the mechanism of HE in humans. Meanwhile, a minority of HE cases is due to anticoagulants, meaning that this kind of model fails to mimic most cases of HE owing to vascular injury caused by basic diseases such as hypertension and diabetes

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mellitus. Moreover, specific criteria have been determined for defining HE in the clinic based on morphological and functional changes [8, 9]. Nevertheless, animal models of HE only demonstrate numerical expansion, while no criteria for judgment have been established.

Also, the mechanisms underlying HE remain unknown. Since the laboratory parameters representing coagulation, inflammation, and microvascular integrity can predict HE, these three aspects may be the predominant mechanisms [4, 10, 11]. As a critical pathophysiological change after ICH, blood–brain barrier (BBB) disruption is considered to be closely associated with HE [12]. However, limited by the absence of an appropriate animal model, it is still controversial whether HE leads to BBB disruption or BBB disruption results in HE, or both.

Aquaporin-4 (AQP4) is the predominant water channel in the brain; it is located on cell surfaces of the BBB and cerebrospinal fluid–brain barrier such as astrocyte foot processes and ependymal cells [13]. AQP4 promotes the formation of cytotoxic brain edema, while accelerating the elimination of vasogenic brain edema [14]. Meanwhile, it has been demonstrated that AQP4 deletion aggravates brain edema following ICH [15]. Moreover, the presence of AQP4 protects against hemorrhagic BBB disruption [16]. Besides this association between BBB disruption and HE, peri-hemorrhagic edema is also independently associated with HE [17]. Connexin43 (Cx43) is the most abundant astrocytic gap junction, enabling small molecules and ions to pass through. It has been reported that Cx43 is associated with hemorrhagic BBB disruption [18]. In addition, Cx43 is also involved in brain edema regulation [19]. Importantly, Cx43 is closely associated with AQP4. Therefore, we speculated that the interaction between AQP4 and Cx43 is critical to HE formation by regulating BBB permeability.

In this study, we generated an ICH model by autologous blood injection, and HE was induced by hypertension and hyperglycemia. Then we determined the criterion for this HE model based on changes of neurological function. We also examined the relationship between HE and BBB disruption and investigated whether this effect was dependent on AQP4 and Cx43 using AQP4 knock-out (KO) mice and multiple Cx43 inhibitors.

Materials and Methods

Animals and Experimental Groups

Male 3–4 month-old wild-type (WT) and AQP4-KO mice weighing 25–33 g were kindly provided by Dr. Gang Hu, Nanjing Medical University, China. Mice were housed under standard laboratory conditions (temperature, 22 ± 2 °C; humidity, 40%) on a 12 h/12 h light–dark

cycle with food and water freely available. The animal studies and protocol were approved by the Institutional Animal Care and Use Committee of Fudan University (Approval No. 20160963A281). Experiments are reported in compliance with the ARRIVE guidelines.

WT mice were randomly divided into 11 groups: Groups 1. Sham operation; 2, ICH; 3, HE; 4, HE plus vehicle (normal saline); 5, HE plus BB-94 (Sigma–Aldrich, St. Louis, MO, USA), a BBB protector; 6, HE plus lipopolysaccharide (LPS, Huaxia Reagent Co., Ltd, Chengdu, Sichuan, China), a BBB disruptor; 7, HE plus carbenoxolone (CBX, Sigma–Aldrich), an inhibitor of gap junctional intercellular communication; 8, HE plus Dynasore (Sigma–Aldrich), an inhibitor of Cx43 redistribution; 9, HE plus Ro318220 (Sigma–Aldrich), a protein kinase C (PKC) ϵ inhibitor; 10, HE plus scrambled small interfering RNA (siRNA); and 11, HE plus Cx43 siRNA.

AQP4 KO mice were randomly divided into 7 groups: Groups 1, HE; 2, HE plus vehicle (normal saline); 3, HE plus CBX; 4, HE plus Dynasore; 5, HE plus Ro318220; 6, HE plus scrambled siRNA; and 7, HE plus siRNA. Each group included 6 mice.

HE Model and Drug Injection

Mice were anesthetized by isoflurane *via* a mask (3% for induction, 1%–1.5% for maintenance in 70% nitrous oxide and 30% oxygen) and the ICH model was made by injection of autologous whole blood, as in our previous work [16]. The HE model was induced by hypertension and (or) hyperglycemia. Hypertension was induced using angiotensin II (Ang II)-releasing osmotic mini-pumps. Each mouse was anesthetized with isoflurane and an osmotic mini-pump (Alzet, Durect Corp., Cupertino, CA, USA) containing Ang II (1000 ng/kg/min) was subcutaneously implanted. Ang II was infused at 11 μ L/h for 14 days [20]. The systolic blood pressure of conscious mice was measured by a noninvasive, computerized tail-cuff method (BP-2010A, Softron Biotechnology, Beijing, China). The mice were first trained for 7 days and then the measurement started 4 days before implantation of the pump. Intraperitoneal injection of 50% dextrose (8 mL/kg) immediately after ICH was used to induce hyperglycemia (normal saline as control) [12]. To determine the optimized HE model, we compared the stability among the three models by measuring hypertension alone, hyperglycemia alone, and hypertension combined with hyperglycemia at 3, 6, 12, 24, and 72 h after ICH. We also recorded the time point at which the hematoma expanded most strongly.

BB-94 (30 μ g/g) and LPS (3 μ g/g) were injected intraperitoneally 1 day before ICH and daily afterward until decapitation. The drugs CBX (1.5 μ g/g), CMP (3.5 μ g/g), Dynasore (50 ng/g), and Ro318220 (4 ng/g)

were intracerebroventricularly injected immediately after ICH under stereotaxic guidance at 0.1 mm from bregma; 0.9 mm lateral; 3.1 mm ventral. The drug doses were in accordance with our preliminary experiments and previous studies [21–23].

SiRNA

SiRNA was prepared based on the literature [23]. Cx43 siRNA or scrambled siRNA was intracerebroventricularly infused at 4 μ L/mouse 1 day before ICH.

Assessment of Lesion Volume

Two methods were used to assess lesion volume.

Coronal Cryosections. After anesthesia, each mouse was transcardially perfused with 15 mL saline. The brain was removed and frozen in isopentane at -20°C . Coronal cryosections (40 μ m thick) were then cut at 400 μ m intervals and scanned at 300 dpi. The areas of hematoma were encircled and measured using ImageJ. The total lesion volume (mm^3) was calculated by multiplying the sum of hemorrhagic areas in each cryosection by the interval between sections [24].

Magnetic Resonance Imaging (MRI) Mice were anesthetized and placed in an MRI mouse fixator. Serial 1.0-mm-thick T2* images were obtained using GE Discovery MR750 3.0T Scanner (Boston, USA) with a dedicated small-animal coil. T2WI images were obtained by using fast spin-echo scan (TR/TE, 4000/96 ms; FOV, 60 $\times$ 60 mm; layer thickness, 1.8 mm; pitch, 0.2 mm; matrix, 256 $\times$ 256, NEX, 1). Hematoma size was subsequently measured by an examiner blinded to the groups using ImageJ (National Institutes of Health, Bethesda, USA). To determine total lesion volume, the measured hypointense areas and thickness of sections were integrated [25]. A total of 140 WT and 93 AQP4-KO mice underwent MRI measurement, including experiments to determine the criterion and the subsequent experiments to test whether HE met the criterion.

Assessment of Hemorrhagic Volume

Each mouse was anesthetized, transcardially perfused, and the ipsilateral hemisphere removed. Hemorrhagic volume was calculated by means of Drabkin's reagent (160 μ L, Sigma–Aldrich) and a spectrophotometer using Liew's protocol [26].

Neurological Testing

Neurological function was evaluated using a combination of behavioral tests as reported previously [27]. In this

scoring system, a normal score was 0 and the maximal score was 12. The higher the score, the worse the neurological function. An observer who was blinded to the groups carried out the tests.

Survival Rate

We observed the mice daily and recorded the cumulative mortality until 3 days after ICH. Then we generated Kaplan–Meier survival plots using a log rank test with GraphPad Software (San Diego, CA, USA). $P < 0.05$ was considered significant [28].

Determination of the Criteria for the HE Model

We first dynamically measured the lesion volumes of each mouse using MRI at 3 h and the optimized time point when the hematoma expanded most after ICH. The percentage of HE was then calculated. We ran neurological tests at the two time points in each mouse and an increased score ≥ 2 (at least one neurological deficit emerged) was regarded as a significant HE. A total of 50 mice were evaluated and the critical value was determined *via* receiver operating characteristic (ROC) analysis.

Determination of BBB Permeability Using Evans Blue

Evans blue (EB, Sigma–Aldrich) was intravenously injected and 1 h later the mice were anesthetized and perfused. Brain tissue samples surrounding the hematoma were acquired as in our previous study [15]. EB content ($\mu\text{g/g}$) was calculated by spectrophotometry and a standard curve was constructed as previously described [29].

Brain Water Content

The method for measuring brain water content was as described in a previous report. The formula is as follows: Brain water content (%) = (wet weight–dry weight)/wet weight $\times 100\%$ [15].

Western Blot

Western blotting was performed as reported previously [30]. The primary antibodies were anti-AQP4 (1:1000; Sigma–Aldrich), anti-occludin (1:1000; Millipore, Billerica, MA, USA), anti-zonula occluden-1 (ZO-1) (1:1000; Millipore), and anti-claudin-5 (1:1000, Millipore) rabbit polyclonal antibodies.

Electron Microscopy

The preparation of brain tissue and electron microscopy were all as in our previous study [31].

Statistical Analysis

All data are expressed as the mean $\pm$ standard deviation and statistical analysis was performed using SPSS22.0. After testing the normality and homogeneity of variance, statistical comparisons among multiple groups were made by using one-way analysis of variance (ANOVA). In case of significant differences identified by ANOVA, intergroup comparisons were performed using Tukey's honestly significant difference test. Comparisons between WT and AQP4-KO mice in each group were carried out using two-tailed Student's *t* tests. The ROC analysis was applied to determine the critical value of the percentage of HE for predicting an increase of neurological score. $P < 0.05$ was considered statistically significant. The statistician who conducted the analyses was blinded to the groups.

Results

Determination of the Optimized Model

The parameters of blood pressure and blood glucose levels of the three HE models are shown in Table 1. We selected three methods for evaluating hematoma volume from morphometric and spectrophotometric measurements. The coronal cryosections at each time point of the three HE models are shown in Fig. 1A. The data from coronal cryosections showed that HE only appeared at 12 h and

24 h after ICH in the hypertension group. In the hyperglycemia group, HE was detected at 24 h and 72 h after ICH. However, HE in the hypertension combined with hyperglycemia group started at 3 h and lasted at least 72 h after ICH (Fig. 1B). MRI images revealed that HE was only observed at 12 h in the hypertension alone as well as at 6 h and 24 h after ICH in the hyperglycemia group, while at each time point in the combined hypertension and hyperglycemia group (Fig. 1C). Hemorrhagic volume assessed by hemoglobin content disclosed that hypertension alone only induced HE at 12 h after ICH and hyperglycemia alone induced HE at 24 h and 72 h. However, HE induced by hypertension and hyperglycemia occurred from 3 h after ICH and lasted 72 h (Fig. 1D). Similarly, the neurological scores were only higher at 12 h and 24 h with hypertension alone as well as at 12 h, 24 h, and 72 h with hyperglycemia alone, while at all time points in the combined group (Fig. 1E). Moreover, survival curves revealed no marked difference in survival rate among the three groups at the end of 72 h (Fig. 1F). Therefore, HE was found in all time points and the three detection methods were consistent in the model induced by hypertension and hyperglycemia (HH-HE model), in which neurological function was in accord with HE and the survival rate was hardly influenced. As a result, we concluded that this HH-HE model is optimal. Moreover, as the difference of hematoma volume between the ICH and HE groups peaked at 24 h after ICH, we selected this time point for subsequent research.

Determination of the Criteria for HH-HE Model

The percentage of HE in the hematoma volume at 3 h and 24 h after ICH in the same mouse was calculated by MRI

Table 1 Blood pressure and blood glucose levels in the three HE models

	0 h	3 h	6 h	12 h	24 h	48 h
Ang II						
Systolic blood pressure (mmHg)	161.5 $\pm$ 1.2	164.6 $\pm$ 1.1	168.7 $\pm$ 1.3	170.2 $\pm$ 1.3	169.1 $\pm$ 1.2	167.5 $\pm$ 1.1
Diastolic blood pressure (mmHg)	112.1 $\pm$ 1.1	114.4 $\pm$ 1.2	117.5 $\pm$ 1.0	120.3 $\pm$ 1.2	118.8 $\pm$ 1.1	116.7 $\pm$ 1.3
Blood glucose (mmol/L)	5.3 $\pm$ 1.1	6.1 $\pm$ 1.2	7.7 $\pm$ 1.6	8.1 $\pm$ 1.3	7.0 $\pm$ 1.5	6.4 $\pm$ 1.2
GluGlu						
Systolic blood pressure (mmHg)	102.3 $\pm$ 0.8	104.1 $\pm$ 0.7	109.4 $\pm$ 1.1	107.6 $\pm$ 1.0	108.3 $\pm$ 0.9	105.2 $\pm$ 0.8
Diastolic blood pressure (mmHg)	67.2 $\pm$ 0.7	70.1 $\pm$ 0.9	74.3 $\pm$ 0.7	72.4 $\pm$ 0.7	71.2 $\pm$ 0.8	70.2 $\pm$ 0.8
Blood glucose (mmol/L)	5.5 $\pm$ 1.2	20.6 $\pm$ 3.6	16.4 $\pm$ 2.2	11.5 $\pm$ 1.8	7.6 $\pm$ 1.3	6.3 $\pm$ 1.5
Ang II + Glu						
Systolic blood pressure (mmHg)	162.3 $\pm$ 1.1	166.4 $\pm$ 1.0	165.7 $\pm$ 1.3	169.2 $\pm$ 1.2	171.2 $\pm$ 1.4	166.8 $\pm$ 1.2
Diastolic blood pressure (mmHg)	113.8 $\pm$ 1.2	115.4 $\pm$ 1.2	117.1 $\pm$ 1.0	119.6 $\pm$ 1.2	121.7 $\pm$ 1.3	116.6 $\pm$ 1.1
Blood glucose (mmol/L)	5.7 $\pm$ 1.3	22.6 $\pm$ 3.3	17.5 $\pm$ 2.4	12.1 $\pm$ 1.7	7.9 $\pm$ 1.4	6.5 $\pm$ 1.3

Ang, angiotensin; Glu, glucose

Fig. 1 Determination of the optimal HE model. **A** Coronal cryosections at each time point from the three HE models. **B** Lesion volume calculated by the coronal cryosection method ($*P < 0.05$ vs ICH). **C** Lesion volume evaluated by MRI scan ($*P < 0.05$ vs ICH). **D** Hemorrhagic volume determined by hemoglobin content ($*P < 0.05$ vs ICH). HE was detected in the hypertension combined with hyperglycemia group from 3 h after ICH and lasted at least 72 h.

scan (Fig. 2A). We listed the percentages of HE and the corresponding presence of an increase in neurological score ≥ 2 , and then established an ROC curve. The area under the curve (AUC) of this ROC curve was 0.763 and the 95% confidence interval (CI) was 0.631–0.896 ($P = 0.001$), indicating the percentage was appropriate for diagnosing changes in neurological function. The cut-

All the three methods were consistent. **E** Neurological scores were higher in the HH-HE group than in the ICH group from 3 h to 72 h ($*P < 0.05$), while higher scores did not occur at all time points in the other two models. **F** Kaplan–Meier plots of survival curves, showing the survival rates in the three HE groups did not differ at the end of 72 h.

off representing the optimized percentage of HE was 45.0% with the sensitivity of 76.9%, specificity of 72.0%, and Youden index (sensitivity + specificity – 1) 0.489 (Fig. 2B). As a result, we concluded that the criterion for this HH-HE model was hematoma volume expansion $\geq 45.0\%$. Only the model in which HE met this criterion was included in the subsequent study.

Fig. 2 **A** MRI scans displaying hematomas (arrows) at 3 h and 24 h after ICH in the same mouse. **B** ROC curve for determining the criterion for the HE model.

Changes of BBB in HH-HE Model

We used the EB method to evaluate the function of the BBB. This demonstrated that the amount of EB extravasation was significantly higher in the HH-HE group than in the ICH group (Fig. 3A). Also, the brain water content was higher in the HH-HE group than in the ICH group (Fig. 3B). The astrocytes and capillary endothelial cells of the normal BBB were regular without swelling. The morphology of tight junctions (TJs) was like lines of electron-dense zones. After ICH, electron micrographs showed astrocyte swelling. In the HH-HE group, more severe swelling of astrocytes and opening of TJs were observed (Fig. 3C). All three TJ proteins decreased after ICH. The expression of ZO-1 and occludin was further down-regulated in the HE group (Fig. 3D).

Effects of BBB Regulator on HE

After administration of the BBB protector BB-94 in the HH-HE model, the hematoma volume was significantly decreased. However, LPS, a BBB disruptor, induced a further expansion of the hematoma (Fig. 3E).

Effects of AQP4 on Neurological Function and BBB Disruption in the HH-HE Model

AQP4-KO mice (Fig. 4A) barely showed any band in Western blots (Fig. 4B). AQP4 was significantly higher in the HH-HE group than in the ICH group (Fig. 4C). In the HH-HE model, AQP4-KO mice had higher neurological scores than WT mice (Fig. 4D). Moreover, survival curves revealed that the survival rate of the KO HH-HE group was 75.0% at the end of 72 h, which was lower than the 91.7% in the WT HE group (Fig. 4E). In evaluation of BBB disruption, more EB extravasation was detected in the KO HH-HE group (Fig. 4F). Meanwhile, a larger hematoma volume was found in AQP4-KO mice by the coronal cryosection method (Fig. 4G). Electron micrographs showed the presence of AQP4 suppressed TJ opening and astrocyte swelling (Fig. 4H).

Effects of Cx43 on HE and BBB Disruption

In the WT HH-HE group, AQP4 was up-regulated by CBX, RO318220, and Cx43 siRNA (Fig. 5A). Meanwhile, these three compounds markedly reduced the hematoma volume as measured from coronal cryosections (Fig. 5B, C). Moreover, EB extravasation was reduced by the inhibitors CBX, RO318220 and Cx43 siRNA, but not by Dynasore and scrambled siRNA (Fig. 5D). As to the ultrastructural changes, electron micrographs showed that TJ opening was blocked by CBX, RO318220, and Cx43 siRNA but not by Dynasore and scrambled siRNA (Fig. 5E). However, all the inhibitors failed to elicit the above effects in the KO HH-HE group (Fig. 6).

Discussion

As noted in the introduction, limitations remain in the current HE models. It is known that the incidence of hypertensive ICH is higher than ICH associated with anti-coagulation. Thus, the majority of HE develops from hypertensive ICH with the feature of basic vascular injury. Unfortunately, current ICH models fail to simulate spontaneous ICH in the clinic, which further makes it hard to find an ideal HE model. Therefore, our work aimed to create a repeatable and comparable HE model with pathophysiological mechanisms close to HE. We used the autologous blood method to create an ICH model, which caused an immediate hematoma with reproducible volume similar to spontaneous ICH in humans. The possible mechanism of immediate hyperglycemia to cause HE is impairment of the integrity of vessels close to the site of initial bleeding as well as increasing the expression of nuclear factor-kappa B and matrix metalloproteinases-9

Fig. 3 Changes in the BBB and effects of BBB regulators in the HH-HE model. **A** EB extravasation is increased in the HH-HE group ($*P < 0.05$). **B** Brain water content is higher in the HH-HE group than in the ICH group ($*P < 0.05$). **C** In the sham group, capillary endothelial cells and astrocytes are regular without swelling. TJs appear as a series of electron-dense zones (arrows). After ICH, electron micrographs show astrocyte swelling (asterisks). In the HH-HE group, swelling of astrocytes (asterisks) is more severe and TJs appear to be modified owing to the presence of detachments of

external plasma membrane leaflets (arrowheads). Right images, magnification of the regions in red on the left image. Scale bars, 2 μm (left); 0.2 μm (right). **D** Western blots showing all the three TJ proteins were decreased after ICH. The expression of occludin and ZO-1 was further down-regulated in the HE group ($*P < 0.05$). **E** The BBB protector BB-94 decreased hematoma volume as measured from coronal cryosections, while the BBB disruptor LPS induced a further expansion of the hematoma in the HH-HE model ($*P < 0.05$).

[12]. To eliminate the hyperosmotic effect of dextrose, we compared the role of mannitol and demonstrated that 50% mannitol failed to induce HE (Fig. S1), suggesting that a hyperosmotic effect did not participate in HE formation in our study. However, the majority of HE patients suffer from hypertension other than diabetes mellitus. Therefore, we selected three HE models: hypertension, hyperglycemia, and both combined and determined the optimal model. Moreover, three methods were used to evaluate hematoma volume, including morphometry and spectrophotometry. The determination of hemoglobin content by spectrophotometry is suitable for measuring the bleeding volume. However, the hematoma volume (lesion volume) detected by CT scan can be influenced by the swelling of tissue, disruption of hemoglobin, and multiple inflammatory mediators produced immediately after bleeding [26]. Therefore, we also selected morphometry using coronal cryosections and MRI scans. The three methods

were consistent and HE was found at all time points in the HH-HE model, so we regarded it as the optimal model. Many patients with ICH only suffer from hypertension, while the hypertension in our model was induced within 14 days, which may be insufficient to cause long-term vascular injury like that in humans. Moreover, in the clinic, a history of hypertension and the blood pressure at ICH onset cannot independently predict HE [1]. Also, intensive blood pressure lowering is unable to alleviate HE [32]. Therefore, although the blood pressure of the mice was elevated in the hypertension alone model, and may aggravate the damage of surrounding vessels after autologous blood injection, stable HE failed to form. Similar circumstances were seen in the results of acute hyperglycemia alone, although diabetes augments the risk of HE [33]. As a result, the optimal HE model includes both short-term basic vascular injury induced by hypertension and stress vessel rupture induced by acute hyperglycemia

Fig. 4 Effects of AQP4 on neurological function and BBB disruption in the HH-HE model. **A** AQP4 KO mice. **B** Western blots showing barely any band in AQP4-KO mice. **C** AQP4 is higher in HH-HE WT mice than in ICH group ($*P < 0.05$). **D** The neurological scores are higher in AQP4-KO mice than in WT mice ($*P < 0.05$ vs WT ICH; $^{\#}P < 0.05$ vs WT HH-HE). **E** Survival curves showing that the survival rate of the KO HH-HE group is 75.4% after 72 h, lower than the 95.8% in the WT HH-HE group. **F** More EB extravasation occurs

in the KO HH-HE group ($*P < 0.05$). **G** AQP4-KO mice have a larger hematoma volume as measured by coronal cryosections than WT mice in the HE model ($*P < 0.05$). **H** Electron micrographs showing that AQP4 KO mice have more TJ opening (arrowheads) and astrocyte swelling (asterisks) than WT mice. Right images, magnification of the region in red on the left image. Scale bars, 2 μ m (left); 0.2 μ m (right).

and elevated blood pressure, which is closer to the pathophysiology of HE in the clinic.

Although no standard definition of HE has been established in patients with ICH, several accepted criteria have been determined in clinical practice, such as $> 33\%$ growth, > 6 mL growth and $> 33\%$ growth, > 12.5 mL [2, 34]. These criteria are dependent on morphological and functional changes. However, such criteria are absent in previous HE animal models, which only demonstrate

numerical expansion. In ICH patients, the definition of HE depends on the change of hematoma volume between the initial and follow-up CT scans. The time of “initial CT scan” is uncertain. Although no research has investigated the optimal time of initial CT scan for HE, our previous study revealed that an initial CT scan with hypodensities performed 1.5–3.0 h after ICH onset better predicts secondary neurological deterioration [35]. In the current work, we demonstrated that, in the HH-HE model,

Fig. 5 Effects of Cx43 on HE and BBB disruption in WT mice. **A** AQP4 is up-regulated by CBX, RO318220, and Cx43 siRNA ($*P < 0.05$ vs WT HH-HE vehicle). **B**, **C** Hematoma volume measured from coronal cryosections is markedly reduced by CBX, RO318220 and Cx43 siRNA ($*P < 0.05$ vs WT HH-HE vehicle).

D EB extravasation is attenuated by the inhibitors containing CBX, RO318220 and Cx43 siRNA but not by Dynasore ($*P < 0.05$ vs WT HH-HE vehicle). **E** Electron micrographs showing that TJ opening (arrowheads) is blocked by CBX, RO318220, and Cx43 siRNA. Scale bar, 0.2 µm.

Fig. 6 Effects of Cx43 on HE and BBB disruption in AQP4-KO mice. None of the inhibitors (CBX, Dynasore, RO318220, and Cx43 siRNA) had an effect on lesion volume measured from coronal cryosections and BBB disruption in the HE model. **A** Coronal

cryosections. **B** Statistical comparison of the lesion volume. **C** EB extravasation. **D** Electron micrographs. Arrowheads, TJ opening; scale bar, 0.2 µm.

stable HE occurred from 3 h after ICH and peaked at 24 h, thus the difference between 3 and 24 h represents the maximal change of hematoma volume. As a result, we selected these time points for determining the criterion. Furthermore, we focused on the changes of neurological function. The battery of behavioral tests contained 6 items (2 scores each), so an increase of score ≥ 2 was equal to the emergence of at least one item. In addition, the application of MRI scans facilitated dynamic measurement of the percentage of HE in the same mouse. Then we determined the criterion by using ROC analysis. The AUC (0.763) and 95% CI (0.631–0.896) indicated a well-established diagnostic model. Meanwhile, the cut-off of the ROC curve was 45.0% with an optimal sensitivity and specificity, suggesting that the expansion of hematoma volume by 45.0% better predicts changes in neurological function. As a result, we concluded that the criterion for this HH-HE model was hematoma volume expansion $\geq 45.0\%$, which may be reliable and stable for experimental use.

The relationship between HE and BBB disruption is still unknown. Relying on the novel HH-HE model and the corresponding criterion, we investigated the underlying mechanisms. In the current study, we made a comprehensive assessment of BBB disruption following HH-HE from several different aspects, comprising morphology and function. We measured EB extravasation to assess BBB permeability of macromolecules, while we also assessed BBB morphologic alterations by electron microscopy with emphasis on TJs by measuring the expression of TJ proteins. TJ proteins such as occludin, ZO-1, and Claudin-5 are crucial for maintaining the function of TJs, an important component of the BBB. The down-regulation of TJ proteins indicates BBB disruption [36]. The results of the current work included increased EB extravasation, the destruction of ultrastructure, and decreased occluding and ZO-1, suggesting that HE results in BBB disruption. In the clinic, HE is largely attributed to the rupture of vessels surrounding the initial hematoma, which can be supported by the CT angiography spot sign [37, 38]. Importantly, BBB dysfunction easily leads to vessel rupture and precedes ICH [39]. Meanwhile, it has been reported that serum markers of BBB remodeling and fibrosis predict HE [11, 40]. To investigate the role of BBB disruption on HE, we resorted to BBB regulators. BB-94 is a matrix metalloproteinases inhibitor, which has been demonstrated to prevent BBB leakage [41]. Meanwhile, LPS affects multiple BBB functions and is used to damage the BBB. The data revealed that BB-94 reduced hematoma volume and LPS exacerbated HE, suggesting that BBB disruption is a promoter of HE [22]. Taken together, we conclude that a reciprocal causal relationship exists between HE and BBB disruption.

Since the HH-HE model is regarded as reliable and stable, finding possible targets for HE prevention is crucial. In view of the above mechanism, we referred to AQP4 and Cx43, which are helpful for BBB protection. Hypertension in our model was induced by Ang II injection, which is reported to activate the extracellular signal-regulated kinase (ERK) pathway in the brain [42]. Importantly, our previous studies showed that activating the ERK pathway up-regulates AQP4 and Cx43 expression [15, 19]. Similarly, hyperglycemia induced by glucose injection also leads to ERK pathway activation [43]. Therefore, it is possible that hypertension and hyperglycemia in our model affect the AQP4 and Cx43 expression *via* the ERK pathway. Moreover, AQP4 regulates the functions of astrocytes due to its distribution, which makes it necessarily important for the maintenance of BBB integrity [44]. It has been emphasized that AQP4 is critical for the maintenance of BBB integrity in developing individuals and adult CNS injuries [45, 46]. Meanwhile, our previous work demonstrated that AQP4-KO aggravates the opening of TJs and the swelling of endothelial cells after ICH [16]. We further investigated the roles of AQP4 in HE using AQP4-KO mice. We found that the presence of AQP4 reduced the neurological deficits, mortality rate, hematoma volume, and BBB disruption in the HH-HE model. These findings indicate that AQP4 participates in alleviating HE due to the maintenance of BBB integrity.

Cx43 is another important channel protein in the brain and it is associated with hemorrhagic BBB disruption [18]. However, as Cx43 has controversial effects in CNS diseases such as cerebral ischemia and tumors [23, 47], and although there has been no research on the relationship between Cx43 and ICH or HE, we speculate that dual effects may occur. More importantly, Cx43 may function in two crucial ways: intracellular redistribution and phosphorylation [48, 49]. Hence, we chose multiple inhibitors to examine the specific actions of Cx43. We disclosed that CBX and Cx43 siRNA reduced hematoma volume and BBB disruption in the HH-HE model, suggesting that Cx43 promotes HE *via* damaging the BBB. Moreover, the role of Cx43 was blocked by the phosphorylation inhibitor RO318220 but not the redistribution inhibitor Dynasore, indicating that Cx43 affects HE *via* phosphorylation. Interestingly, AQP4 was up-regulated by CBX, RO318220, and Cx43 siRNA and all the inhibitors failed to work in AQP4-KO mice. It has been reported that Cx43 is closely associated with AQP4 [50]. Meanwhile, Cx43 and AQP4 have the same upstream and downstream pathways, e.g. PKC and ERK [15, 51–53]. As a result, it is possible that the effect of Cx43 on HE is AQP4-dependent.

Although the optimal model in the current study was the HH-HE model, several limitations are of concern. This

model is based on short-term hypertension and acute hyperglycemia, which partially mimic HE pathophysiology and stably induce HE. However, most ICH patients undergo long-term hypertension with or without long-term diabetes mellitus. The extent of basic vascular injury may differ from our model. Therefore, further studies are needed to compare this HH-HE model with long-term hypertension and/or hyperglycemia HE models, including the stability of HE and the morphology and function of vessels.

Conclusions

The optimized HE model in our study is induced by hypertension and hyperglycemia after autologous blood injection. This model includes both the basic vascular injury induced by hypertension and stress vessel rupture induced by hyperglycemia, which is closer to the pathophysiology of HE in the clinic. The criterion for this HH-HE model is hematoma volume expansion $\geq 45.0\%$, which better represents the change in neurological function. HE leads to BBB disruption and BBB disruption further promotes the process of HE. Moreover, AQP4 participates in alleviating HE due to maintenance of BBB integrity. Meanwhile, Cx43 regulates HE *via* phosphorylation, which is AQP4-dependent. Besides facilitating translational research on HE, the present research provides double targets for protection against BBB disruption following HE, which may help in the development of new medications.

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Conflict of interest The authors declare that they have no conflict of interests.

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