Original Article
Downregulating hypoxia-inducible factor-1α expression with perfluorooctyl-bromide nanoparticles reduces early brain injury following experimental subarachnoid hemorrhage in rats

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Abstract: The aim of the present study was to investigate the effects of perfluorooctyl-bromide (PFOB) nanoparticles on hypoxia-inducible factor 1 alpha (HIF-1α) and its downstream target genes in early brain injury (EBI) after subarachnoid hemorrhage (SAH). Healthy male Sprague Dawley rats (n=100) were randomly divided into five groups: Sham, SAH, SAH + vehicle, SAH + 5 mg/kg PFOB and SAH + 10 mg/kg PFOB. A rat model of SAH was created by endovascular perforation, and PFOB treatment (5 mg/kg or 10 mg/kg injected into the caudal vein) was initiated 1 h after SAH. All rats were subsequently sacrificed 24 h after surgery. Treatment with PFOB significantly alleviated EBI (including neurological dysfunction, brain edema, blood-brain barrier disruption (BBB), and neural cell apoptosis). In addition, it also suppressed the expression of HIF-1α, vascular endothelial growth factor (VEGF) and BNIP3 in the rat hippocampus. The effects of 10 g/kg PFOB were found to be more obvious than those of 5 g/kg PFOB. Our work demonstrated that PFOB treatment alleviated EBI after SAH, potentially through downregulation of the expression of HIF-1α and its target genes, which led to reduced cell apoptosis, BBB disruption and brain edema.

Keywords: Early brain injury, hypoxia-inducible factor 1 alpha, perfluorooctyl-bromide, subarachnoid hemorrhage, vascular endothelial growth factor

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a devastating disease with high morbidity and severe mortality; roughly 12.4% of patients die immediately after SAH, and about 50% of SAH survivors have varying degrees of nerve function loss [1]. Moreover, approximately 30% of patients with aneurismal SAH die within the first few days [2]. An increasing number of studies show that early brain injury (EBI) is the principal cause of high mortality and poor outcomes in patients following SAH. EBI refers to direct damage to the cerebrum within 72 h after SAH, and encompasses all the pathophysiological changes that occur in brain tissue before late-onset cerebral vasospasm (3 days to 3 weeks). Such changes include cerebral edema, blood-brain barrier (BBB) damage, neuronal death, and microcirculatory dysfunction

[3]. Thus, EBI intervention is key to improving the prognosis of SAH.

It has been suggested that global cerebral ischemia, resulting from a dramatic increase in intracranial pressure and fall in cerebral blood flow, may play an important role in EBI [4]. Hypoxia-inducible factor 1 alpha (HIF-1α) is a transcription factor that is expressed in mammals and humans under conditions of ischemia or hypoxia, and can activate multiple downstream genes to regulate the body's response to external stimuli [5]. Numerous studies have reported that HIF-1α can be seen in rat models of SAH in the parenchyma [6-9]. Inhibiting the expression of HIF-1α can relieve the constituents of EBI, including cerebral edema, disruption in BBB permeability, and neuronal apoptosis [6, 11]. HIF-1α acts mainly by activating the downstream genes BNIP3 [6] and vascular

Table 1. Neurobehavioral function deficit score standard

Item	Actions and activities	Grade
Food intake	Finished eating food	0
	Ate a part of the food given	1
	Did not eat	2
Activity	Walked freely	0
	Moved when probed	1
	Little to no movement	2
Functional deficit	No functional deficit	0
	Unsteady gate	1
	Non-ambulatory	2

endothelial growth factor (VEGF) [3], which are activated during EBI after SAH. Therefore, inhibiting the expression of HIF-1α and subsequent downstream activation of VEGF and BNIP3 may offer possible targets for EBI treatment.

Perfluorooctylbromide (PFOB) belongs to the new third-generation perfluorocarbons (PFCs), which have many distinctive properties, such as stability, efficient oxygen properties, low surface tension, and low toxicity. These unique qualities are the foundation of its use as a therapeutic agent, new contrast agent, and targeted drug carrier [12, 13]. By ultrasonic emulsification, PFOB nanoscale particlescan be directly administered viaintravenous injection. These nanoscale PFOB particles are small in diameter, have a long half-life and circulatory time, and easily pass through the endothelial gap [14]. The possible role of PFOB nanoparticles in the treatment of SAH is still being explored, and our previous study yielded preliminary evidence that PFOB nanoparticles have neuroprotective effects against EBI after SAH [15], though the exact underlying mechanisms remain unclear. Based on our previous findings, we proposed that PFOB nanoparticles downregulate the expression of HIF-1α and its downstream target genes, especially VEGF and BNIP3, which may be one of the mechanisms of the neuroprotective effect exerted by PFOB nanoparticles after SAH.

Materials and methods

Animal groups

All protocols for animal experiments (including all surgical procedures) were approved by the Institutional Animal Care Committee of Chongqing Medical University and conformed to the guidelines of the National Institutes of Health on the care and use of animals. We included 100 male Sprague-Dawley rats (300-320 g) obtained from the Experimental Animal Center of Chongqing Medical University in this study. Rats were randomly assigned into five groups: Sham, SAH, SAH + vehicle, SAH + 5 g/kg PFOB, and SAH + 10 g/kg PFOB (n=20 in each group).

SAH model

We adopted an endovascular perforation rat model of SAH that has been described previously [16]. Rats were anesthetized with 10% chloral hydrate (3.5 ml/kg, i.p), then the right external carotid artery (ECA) was exposed, isolated and severed, leaving a stump. The internal carotid artery (ICA) and the common carotid artery (CCA) were clamped via vascular block. The stump of the ECA was cut open, and a blunted 3-0 monofilament nylon suture was inserted for about 18-20 mm until resistance was felt, then insert about another 3 mm, puncturing the artery wall. Sham-operated rats underwent the same steps without perforation. Any rats that died during the process of modeling were replaced in a timely fashion to ensure the presence of 20 animals in each group.

PFOB nanoparticle treatment

PFOB (Alliance Pharmaceutical, USA) was made into emulsified nanoparticles by the Ultrasound Imaging Institute, Chongqing University of Medical Science. Oxygen was dissolved into PFOB nanoparticles to saturation, then one of two doses (5 g/kg and 10 g/kg) of PFOB was administered by tail intravenous injection 1 h after SAH. The SAH + vehicle group were injected with the same volume of saline solution.

Mortality, neurobehavioral functional deficit scores, and SAH severity evaluation

Overall mortality was calculated at 24 h after SAH; rats that did not survive the process were excluded from further assessment. The neurobehavioral function defect score was evaluated at the same time, based on the scoring system proposed by Yanaguchi [17] (scoring criteria are shown in **Table 1**). The severity of SAH was evaluated by the scoring system pro-

posed by Sugawara [18], in which the basal cistern was divided into six segments. Each segment was allotted a grade from 0-3 depending on theamountof subarachnoid blood clotted in that segment as follows: Grade 0: no subarachnoid blood, 1: minimal subarachnoid blood, 2: moderate blood clot with recognizable arteries, 3: blood clotobliterating all arteries within the segment. Depending on the final score animals were divided into three groups as follows 0-7: mild SAH, 8-12: moderate SAH, and 13-18: severe SAH.

Brain water content

Rats (n=5) in each group were anesthetized and decapitated, and their brains were harvested and quickly weighed (wet weight). Brain samples were then placed in an oven at 105°C for 48 h and weighed again (dry weight). Brain water content was calculated using the following formula: [(wet weight-dry weight)/wet weight] × 100% [19].

Determination of BBB permeability

BBB permeability was assessed by Evans Blue (EB) extravasation 24 h after SAH. Briefly, 2% EB (5 mg/kg, Sigma-Aldrich)was injected into the right femoral vein. One hour later, rats were anesthetized andtranscardiallyperfused with PBS to remove the intravascular EB dye. After decapitation, the brains were weighed, homogenized and centrifuged (2000 rpm, 20 min, 4°C); then the supernatant was collected and the procedure repeated three times. The resulting supernatant was measured for absorbance of EB at 620 nm using a spectrophotometer.

Histology and TUNEL staining

Rats were deeply anesthetized with 10% chloral hydrate, and sacrificed by intracardial perfusion with physiological saline and 4% paraformaldehyde. Brains were quickly removed and immersed in 25% sucrose dissolvedin PBS for 48 h. Brains were then frozen and cut into coronal sections 10 μm in thickness. Apoptotic cells were detected using a TUNEL staining kit (POD, Roche Applied Science, USA). Thereafter, sections were incubated with permeabilization solution (10 min, at room temperature),washed with PBS (two times 5 min), incubated in the TUNEL reaction mixture (1 h, at 37°C), and then washed with PBS three times. Cell nuclei were stained byincubating with DAPI for 8 min at room temperature, neutral gum slice observation. Using an Olympus PM20 automatic push around (Japan) observation microscope, the hippocampal CA1 area was visualized and positive cell were dyed green. Image-Pro plus software was used to calculate the average optical density of the TUNEL-positive cells.

RT-qPCR

Total RNA in each group was extracted from the hippocampus using the TRIzol extraction method as described previously. RNA was measured by spectrophotometry and reversed-transcribed into cDNA using the AMV First Strand cDNA Synthesis kit (SK 2445) and CFX96TM Real-Time PCR Detection System. PCR products were synthesized using the TaKaRa Sybr Green PCR Master Mix and analyzed in real-time with the detection system (ABI Prism 7700 Sequence Detection System). The primers used were designed according to the Gene Bank gene sequences, including HIF-1α: forward primer 5'-GTCAGTCACTGCCACCGCAACTG-3', reverse primer 5'-CTGTCTGGTGAGGCTGTCCGA-3', VEGF: forward primer 5'-ACGTCGGAGAGCAA-CGTCAC-3', reverse primer 5'-AGGCTCACAGTG-AATGTGGTCA-3', BNIP3: forward primer 5'-CC-TTTGCGGAGCCACCATGT-3', reverse primer 5'-AGACGGAAGCTGGAACGCTG-3', GAPDH: forward primer 5'-TAGTTGCGTTACACCCTTTCTTG-3', reverse primer 5'-TCACCTTCACCGTTCCAGTTT-3'. The following PCR protocol was applied: 95°C for 60 s, 95°C for 15 s, 60°C for 60 s, 40 cycles. Results were analyzed by the rCt method, which reflects the difference in threshold for the target gene relative to that of GAPDH in each sample.

Immunohistochemistry

Twenty-four hours after SAH induction, each group of rats (n=5/group) was anesthetized and perfused with 4% paraformaldehyde. Brains were removed and post-fixed for 48 h, frozen and thencut into 4 μm coronal sections through paraffin-embedding dehydration. The sections were dewaxed using xylene, rehydrated, run through an antigen repair protocol, blocked for endogenous peroxidase and then incubated in PBS. Rabbit-anti-HIF-1α antibody (1:100, ab5168, Abcam), VEGF (1:100, ab46-154, Abcam), and BNIP3 (1:100, ab109326, Abcam) were added to the sections and incu-
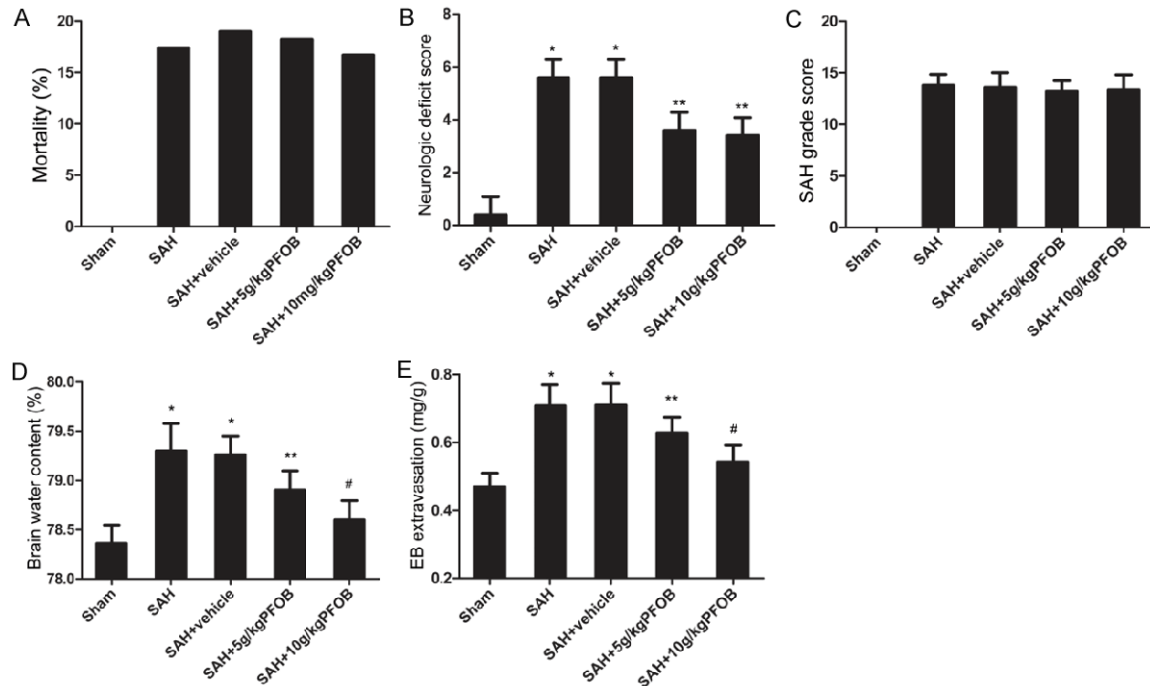
Figure 1. Bar graphs showing the influence of PFOB on mortality (A), neurological deficit scores (B), SAH grade score (C), brain-water content (D), and EB extravasation (E) 24 h after surgery. Although PFOB treatment did not reduce mortality or improve SAH score, it also significantly reduced brain-water content and BBB permeability and improved neurologic function compared with the SAH group. In terms of neurological deficit, brain-water content, and EB extravasation, there was no significant difference between the SAH and SAH + vehicle groups. Values are shown as mean ± SD. *$p< 0.05$ versus sham group; **$p< 0.05$ versus SAH or SAH + vehicle group; #$p< 0.05$ versus SAH + 5 g/kg PFOB group.

bated overnight at 4°C. Sections were then washed three times with PBS for 5 min each and incubated with horseradish peroxide (HRP)-labeled active rabbit-anti-goat IgG working solution at 37°C for 30 min and washed three times with PBS for 5 min each. Sections were then stained for 3-5 min in DAB staining solution, restained in hematoxylin, differentiated using hydrochloric alcohol, and gradient dehydrated. Using an Olympus PM20 (Japan) and TCFY-2050 fully automatic microscope observation biopsy pathology system, we visualized positive sections in which the cytoplasm or nucleus was dyed yellow or brown. Image Pro plus software was used to compute HIF-1α, VEGF, and BNIP3 average optical density values.

Western blots

Anesthetized rats (n=5) were decapitated, brains were removed, and hippocampi were quickly isolated. Hippocampus tissue was homogenized and then centrifuged (12000 *g*,

12 min). The centrifugal supernatant and a quarter of the loading buffer was denatured at 95°C for 10 min. Protein samples (70 µg) were then loaded onto 10% SDS polyacrylamide gel electrophoresed at 100 v for 2 h, and transferred to polyvinylidene fluoride (PVDF) membranes at 110 v for 90 min. Membranes were blocked with 5% nonfat defatted milk buffer for 2 h and incubated overnight at 4°C with the same primary antibodies that were used for immunohistochemistry (HIF-1α 1:1000, VEGF 1:600, BNIP3 1:500), along with rabbit anti-GAPDH antibody 1:1000 (Proteintech). After washing with tris-buffered saline and Tween 20 (TBST), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted at 1:1000 for 1 h at 37°C. The protein band density was detected by Bio-Rad image analysis.

Date analysis

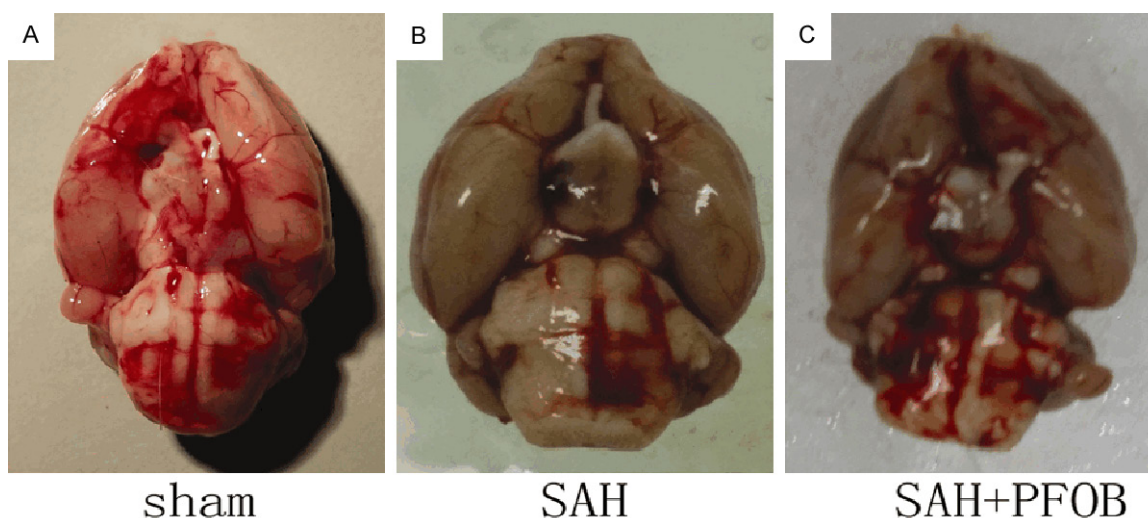All dataare presented as the mean ± SD. IBM SPSS Statistics, version 17.0.0, was used for

Figure 2. Representative images of SAH brain: A. Sham group. B. SAH + vehicle group. C. SAH + PFOB group.

statistical analysis of all data. Statistical significance was verified by one-way analysis of variance (ANOVA) followed by a Tukey test for multiple comparisons. Differences in mortality between groups were tested by a chi-square test. A probability value of $p < 0.05$ was considered statistically significant.

Results

Influence of PFOB on mortality, neurologic deficit scores, and SAH grade

No rats died in the sham-operated group. In the SAH and SAH + vehicle group, the mortality rates were 17.4% and 19.0% respectively. There was a mortality rate of 18.2% in the SAH + 5 g/kg PFOB group and a rate of 16.7% in the SAH + 10 g/kg PFOB group. These mortality rates were not significantly different from those in the SAH and SAH + vehicle groups ($p > 0.05$; **Figure 1A**). Rats in the sham group exhibited almost no neurologic deficits (0.4 ± 0.55). On the other hand, neurological deficit scores in the SAH and SAH + vehicle group were both significantly higher than those in the sham-operated group (SAH, 5.6 ± 0.54; SAH + vehicle, 5.6 ± 0.55, $p < 0.05$), but the SAH and SAH + vehicle group did not differ from each other. Moreover, treatment with PFOB at 5 g/kg and 10 g/kg significantly reduced the neurological deficit scores (SAH + 5 g/kg PFOB, 3.6 ± 0.53; SAH + 10 g/kg PFOB, 3.4 ± 0.51, $p < 0.05$; **Figure 1B**). No subarachnoid blood clots were found in the sham-operated group (0 ± 0), whereas many

blood clots were evident in SAH and SAH + vehicle groups (SAH, 13.8 ± 0.84; SAH + vehicle, 13.6 ± 1.14). Finally, SAH grading scores were higher in the SAH and SAH + vehicle groups than in the sham group. Furthermore, compared with the SAH and SAH + vehicle groups, treatment with PFOB (5 g/kg or 10 g/kg) did not significantly reduce the SAH grade (SAH + 5 g/kg PFOB, 13.2 ± 0.83; SAH + 10 g/kg PFOB, 13.4 ± 1.14, $p > 0.05$; **Figures 1C** and **2**).

Influence of PFOB on brain water content and BBB permeability

Brain edema is common in EBI, and disruption of BBB permeability has been considered an important contributing factor in brain edema. Compared with the sham group (Sham, 79% ± 0.14%, 0.32 ± 0.06), brain water content and EB extravasation were significantly higher in SAH and SAH + vehicle groups (SAH, 79.6% ± 0.08%, 0.687 ± 0.08; SAH + vehicle, 79.65% ± 0.12%, 0.71 ± 0.08; **Figure 1D** and **1E**), whereas administration of PFOB after SAH dramatically reduced brain-water content and EB extravasation in a dose-dependent manner (SAH + 5 g/kg PFOB, 79.4% ± 0.08%, 0.562 ± 0.06; SAH + 10 g/kg PFOB, 79.2% ± 0.08%, 0.43 ± 0.05; **Figure 1D** and **1E**).

Influence of PFOB on cell apoptosis

We have previously used the TUNEL assay to assess apoptosis in experimental models of stroke [19-21]. In the current study, TUNEL
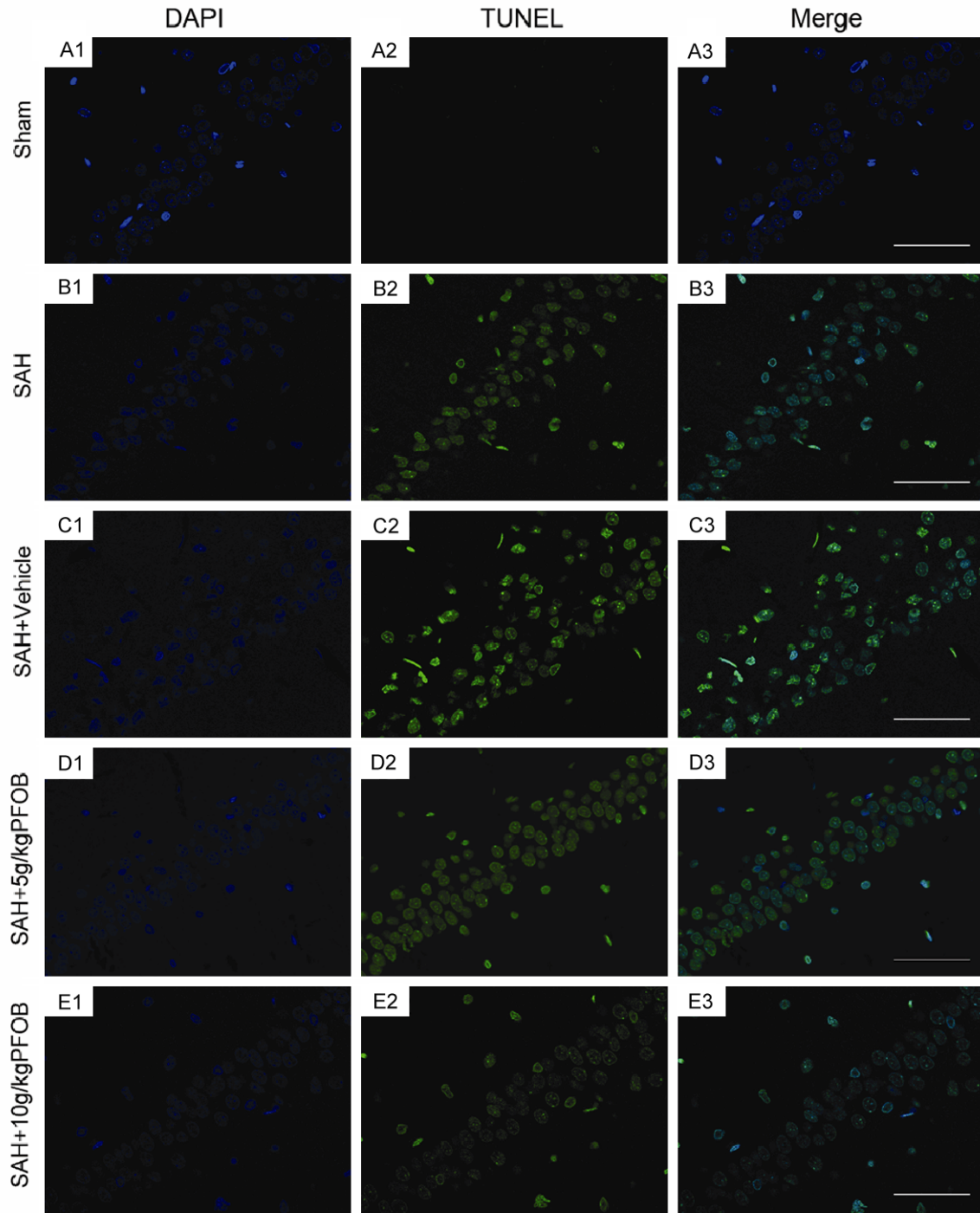
Figure 3. Influence of PFOB on cell apoptosis in the hippocampus CA1 region 24 h following SAH. A-E. Show TUNEL/DAPI photomicrographs in different groups. F. Shows quantification of TUNEL-positive cells in the groups. Compared with the sham-operated group, there were significantly more TUNEL cells in the SAH and SAH + vehicle groups. Administration of PFOB (5 g/kg or 10 g/kg) markedly reduced the number of apoptotic cells after SAH, and this effect was more evident in the 10 g/kg group than in the 5 g/kg group. There was no significantly difference between the SAH and SAH + vehicle groups. Values are shown as mean ± SD. *$p <$ 0.05 versus Sham group; **$p <$ 0.05 versus SAH or SAH + vehicle group; #$p <$ 0.05 versus SAH + 5 g/kg PFOB group. Scale bar: 50 μm.
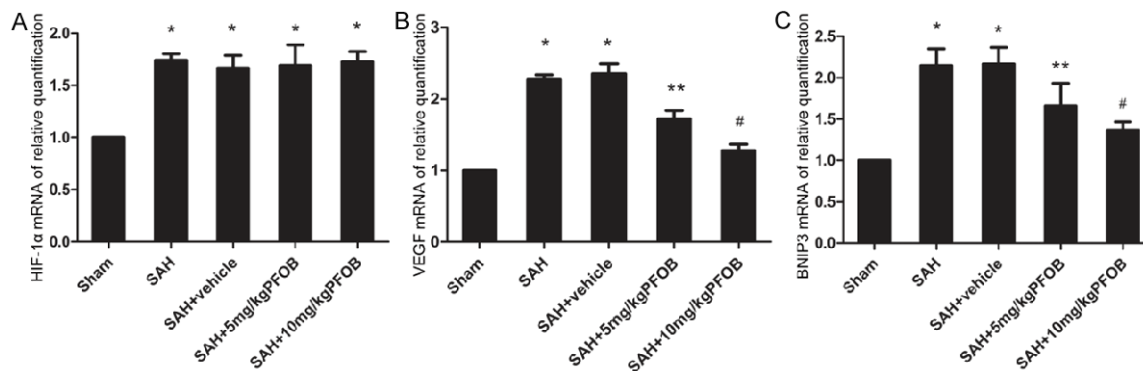
Figure 4. Levels of *HIF-1α, VEGF,* and *BNIP3* mRNA were significantly higher in SAH and SAH + vehicle groups compared with the sham-operated group. Treatment with PFOB did not significantly reduced HIF-1α mRNA (A) but the levels of *VEGF* and *BNIP3* mRNA were dramatically attenuated, in a dose-dependent manner (B, C). There was no significant difference in the expression of *HIF-1α, VEGF, or BNIP3* mRNA between the SAH and SAH + vehicle groups. Values are shown as mean ± SD. *$p < 0.05$ versus Sham group; **$p < 0.05$ versus SAH or SAH + vehicle group; #$p < 0.05$ versus SAH + 5 g/kg PFOB group.

staining showed that rare apoptotic neurons were found in the hippocampus of animals in the sham-operated group at 24 h after surgery (Sham, 0.47 ± 0.15), and that higher numbers of TUNEL-positive neurons were detected in the SAH and SAH + vehicle groups (SAH, 2.53 ± 0.15; SAH + vehicle, 2.77 ± 0.16). Administration of PFOB (5 g/kg or 10 g/kg) significantly decreased the proportion of TUNEL-positive neurons in the hippocampus in a dose-dependent manner (SAH + 5 g/kg, 2.0 ± 0.2; SAH + 10 g/kg, 1.56 ± 0.15; **Figure 3**).

Influence of PFOB on HIF-1α, VEGF and BNIP3 mRNA as assessed by RT-qPCR

Statistical results of RT-qPCR studies are shown in **Figure 4**. Low levels of *HIF-1α, VEGF,* and *BNIP3* mRNA were observed in the sham-operated group (Sham, 1.0 ± 0, 1.0 ± 0, 1.0 ± 0). In comparison, the levels of *HIF-1α, VEGF* and *BNIP3* mRNA significantly increased after surgery in SAH and SAH + vehicle groups (SAH, 1.74 ± 0.03, 2.3 ± 0.06, 2.14 ± 0.08; SAH + vehicle, 1.66 ± 0.05, 2.35 ± 0.05, 2.16 ± 0.08). Treatment with 5 g/kg or 10 g/kg PFOB did not dramatically suppress the expression of HIF-1α mRNA (SAH + 5 g/kg, 1.73 ± 0.04; SAH + 10 g/kg, 1.56 ± 0.05), but the mRNA of some of its downstream target genes (*VEGF* and *BNIP3)* was significantly lower than in the SAH and SAH + vehicle groups. Finally, the effects of 10 g/kg PFOB were more obvious than 5 g/kg PFOB (SAH + 5 g/kg, 1.72 ± 0.06, 1.68 ± 0.1; SAH + 10 g/kg, 1.27 ± 0.05, 1.33 ± 0.06).

Influence of PFOB on HIF-1α, VEGF and BNIP3 as assessed by immunostaining

At 24 h after SAH, the expression of HIF-1α, VEGF and BNIP3 was assessed in hippocampus neurons. More specifically, HIF-1α and VEGF were detected in the nucleus and cytoplasm, while BNIP3 was expressed only in the cytoplasm [6]. Hippocampal tissue from sham-operated group rats showed a few cleaved cells positive for HIF-1α, VEGF, and BNIP3 (Sham, 1.02 ± 0.22, 0.72 ± 0.15, 0.95 ± 0.22; **Figure 5A1**, **5B1** and **5C1**). Compared with the sham group, brain samples obtained from rats after 24 h surgery in the SAH and SAH + vehicle groups showed significantly more HIF-1α-, VEGF-, and BNIP3-positive cells (SAH, 2.3 ± 0.18, 2.67 ± 0.2, 2.7 ± 0.18; SAH + vehicle, 2.2 ± 0.17, 2.68 ± 0.22, 2.82 ± 0.22; **Figure 5A2**, **5A3**, **5B2**, **5B3** and **5C2**, **5C3**). Compared with the SAH and SAH + vehicle groups, treatment with 5 g/kg PFOB (SAH + 5 g/kg PFOB, 1.7 ± 0.18, 2.0 ± 0.20, 2.15 ± 0.23; **Figure 5A4**, **5B4** and **5C4**) or 10 g/kg PFOB (SAH + 10 g/kg PFOB, 1.37 ± 0.09, 1.47 ± 0.17, 1.65 ± 0.2; **Figure 5A5**, **5B5** and **5C5**) 1 h after surgery markedly reduced the number of HIF-1α-, VEGF-, and BNIP3-positive cells in the hippocampal tissue, in a dose-dependent manner.

Influence of PFOB on HIF-1α, VEGF and BNIP3 expression

Western blot analysis of the hippocampus showed that the expression of HIF-1α, VEGF,
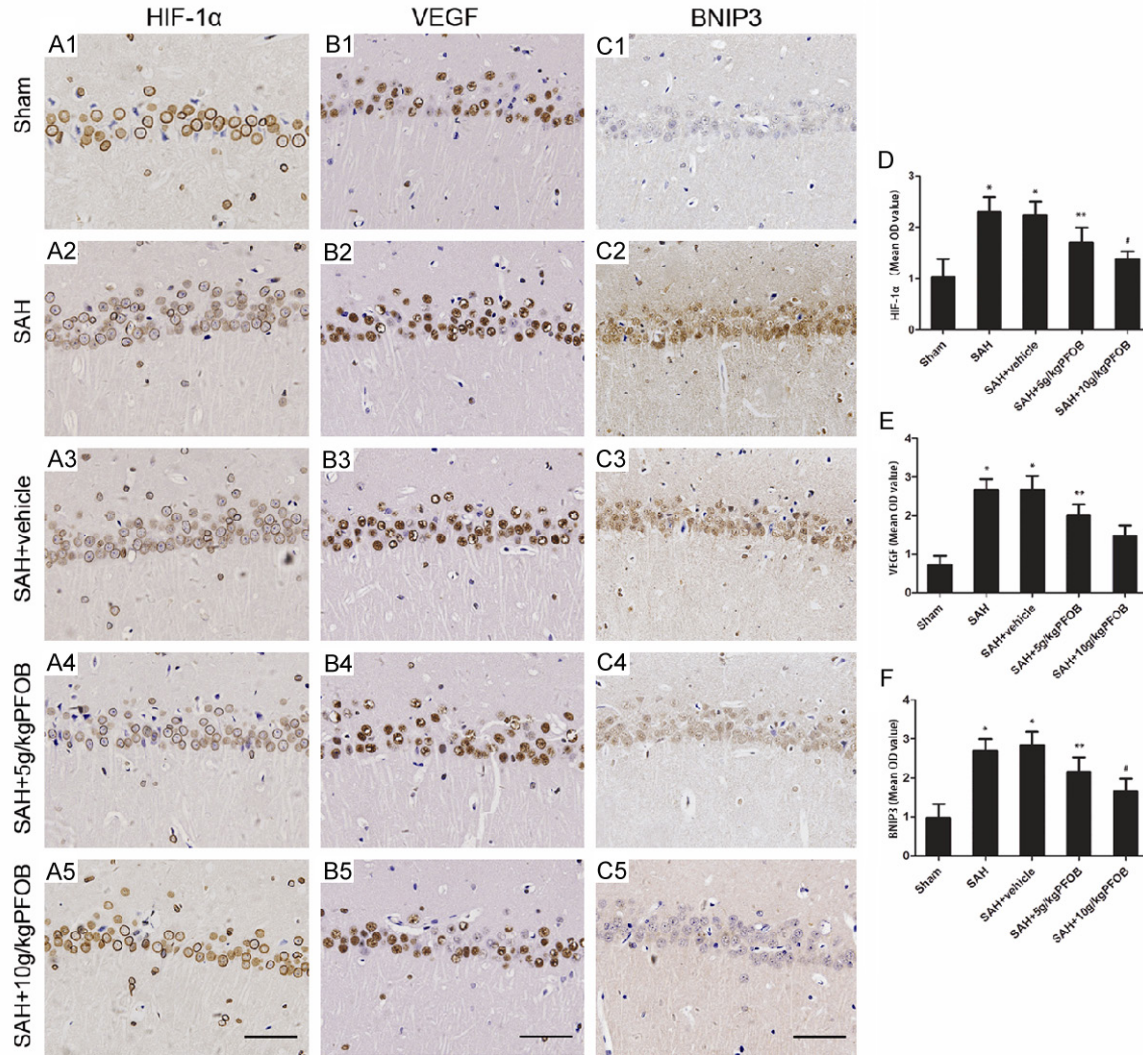
Figure 5. Immunohistochemistry was used to analyze the influence of PFOB on the expression of HIF-1α (A1-A5), VEGF (B1-B5) and BNIP3 (C1-C5) in the hippocampal CA1 region at 24 h following SAH. A1-A5 show HIF-1α expression in all groups, B1-B5 show VEGF expression, and C1-C5 show BNIP3 expression. D-F shows quantification of HIF-1α-, VEGF-, and BNIP3-positive cells respectively in all groups. Compared with the sham-operated group, there are significantly more positive cells in the SAH and SAH + vehicle groups. Administration of PFOB (5 g/kg or 10 g/kg) markedly reduced the number of positive cells after SAH, and this effect was more evident in the 10 g/kg group than in the 5 g/kg group. There was no significantly difference in the expression of HIF-1α, VEGF and BNIP3 between the SAH and SAH + vehicle groups. Values are shown as mean ± SD. *$p < 0.05$ versus Sham group; **$p < 0.05$ versus SAH or SAH + vehicle group; #$p < 0.05$ versus SAH + 5 g/kg PFOB group. Scale bar: 20 μm.

and BNIP3 was low in the sham group 24 h after surgery (Sham, 1.0 ± 0, 1.0 ± 0, 1.0 ± 0). In comparison, the expression of HIF-1α, VEGF, and BNIP3 was significantly higher in the SAH and SAH + vehicle groups (SAH, 2.76 ± 0.06, 2.67 ± 0.11, 1.73 ± 0.08; SAH + vehicle, 2.71 ± 0.08, 2.72 ± 0.13, 1.74 ± 0.08). However, administration of 5 g/kg PFOB or 10 g/kg PFOB 1 h after surgery dramatically suppressed the expression of HIF-1α, VEGF, and BNIP3 in a dose-dependent manner (SAH + 5 g/kg PFOB,

1.81 ± 0.07, 1.49 ± 0.07, 1.38 ± 0.05; SAH + 10 g/kg PFOB, 1.46 ± 0.04, 1.24 ± 0.09, 1.24 ± 0.07; **Figure 6**).

Discussion

Subarachnoid hemorrhage is a common disease and since Zhang [3] et al. first proposed EBI as the main cause of early death following SAH, this view has been increasingly recognized by researchers. EBI is a series of complicated pathophysiologic mechanisms that result
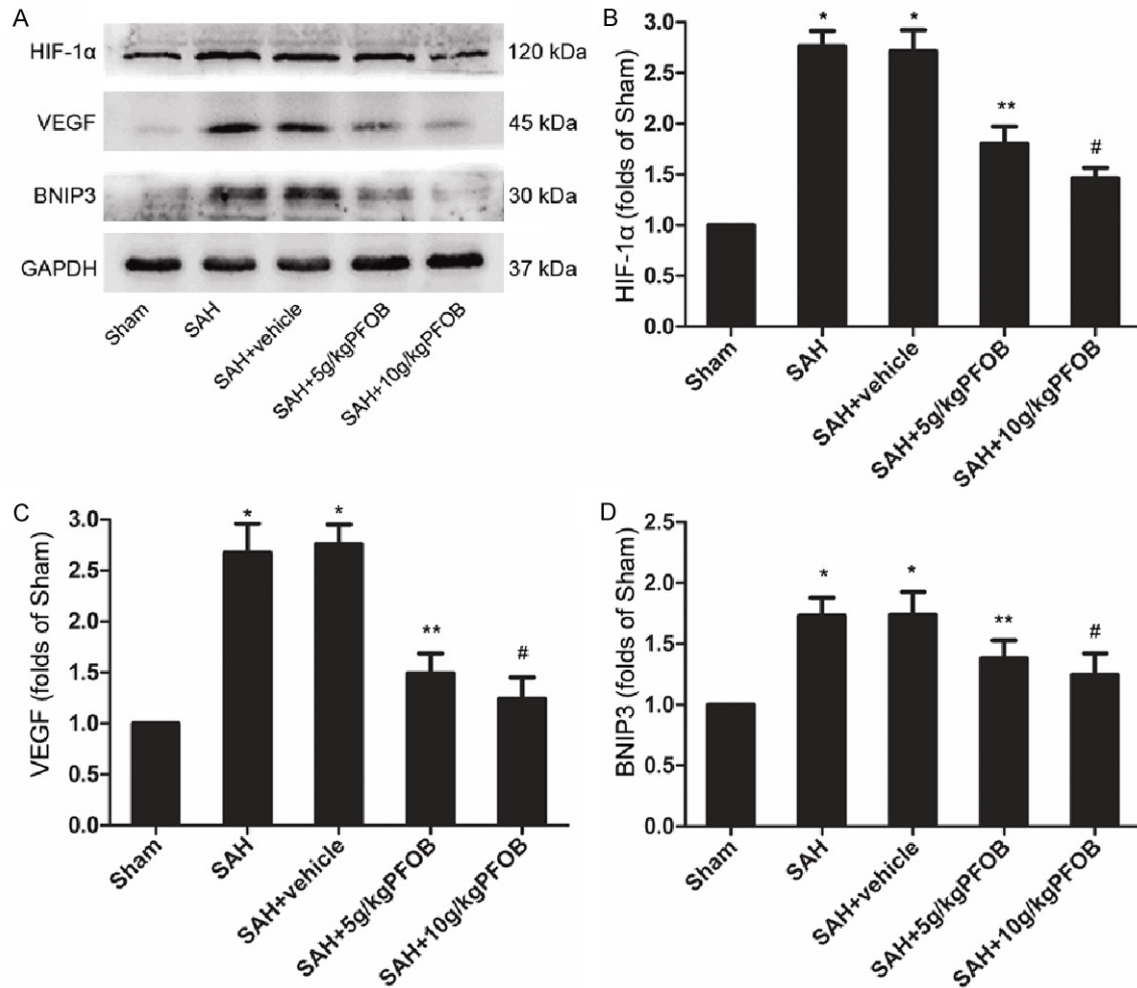
Figure 6. Western blot was used to analyze the influence of PFOB on the expression of HIF-1α, VEGF, and BNIP3 in the hippocampus 24 h after SAH. A. Representative panels showing HIF-1α, VEGF, and BNIP3 protein expressed in all groups. B-D. Showing relative optical densities of HIF-1α, VEGF, and BNIP3 respectively, normalized to GAPDH. Levels of HIF-1α, VEGF, and BNIP3 in the SAH and SAH + vehicle groups were significantly higher than in the sham group. Treatment with PFOB (5 g/kg or 10 g/kg) markedly reduced this up-regulation of protein. There was no significant difference in the expression of HIF-1α, VEGF, and BNIP3 between the SAH and SAH + vehicle groups. Values are shown as mean ± SD. *$p < 0.05$ versus Sham group; **$p < 0.05$ versus SAH or SAH + vehicle group; #$p < 0.05$ versus SAH + 5 g/kg PFOB group.

from SAH. It has been revealed that acute cerebral ischemia, resulting from dramatic changes in intracranial pressure and reduced cerebral blood flow, might play an important role in EBI [22]. Thus, emphasis has been placed on EBI intervention as the key to improving the prognosis of SAH.

In 1992, HIF-1, which is an oxygen-dependent transcription factor, was found in Hep3B cell extracts by Semenza et al. [23]. Specially, they found that HIF-1 reacted with low oxygen elements, leading to downstream gene transcription. HIF-1 consists of an oxygen-regulated α

subunit and a constitutively expressed β subunit; HIF-1α is not only the regulatory subunit, but also the active subunit. Several studies have demonstrated that HIF-1α is closely related to brain injury, cerebral ischemia and cerebral hemorrhagic diseases [24, 25]. Ostrowski et al. [6] first reported the expression of HIF-1α in brain tissue in a rat model of SAH. Moreover, Wang et al. [26] found that HIF-1α protein played an important role in brain edema after SAH via a molecular signaling pathway involving aquaporin-4 and matrix metalloproteinase-9. Hu et al. [27] then described HIF-1α expression in a rat model of SAH, specifically reporting that

HIF-1α significantly increases 12 h after SAH, peaks at 24 h, and then is significantly reduced by 72 h. At the same time, HIF-1α was determined to be involved in neuronal apoptosis associated with EBI. As a result, it was suggested that the inhibition of HIF-1α could play a role in neuroprotection. Interestingly, Ostrowki et al. [6] confirmed that hyperbaric oxygen therapy can reduce EBI after SAH, partly by inhibiting HIF-1α and its downstream effects on VEGF and BNIP3 protein expression. Moreover, Wu et al. [10] reported that the HIF-1α inhibitor 2ME2 can reduce brain edema and neuronal apoptosis associated with EBI without affecting the intracranial pressure, cerebral perfusion pressure, or cerebral blood flow. These studies confirm that inhibiting HIF-1α in different ways can play a protective role against EBI after SAH.

HIF-1α is not directly involved in SAH, but rather it translocates into the nucleus where it combines with HIF-1β and generates HIF-1α, then activates downstream target genes, especially VEGF and BNIP3, and contributes to the development of EBI. Research suggests that up-regulation of VEGF is mainly responsible for damage to the BBB and the deterioration of brain edema in EBI. In addition, suppression of VEGF by HIF-1α inhibitors can reduce brain edema [3, 28]. BNIP3 belongs to a class of proteins known as "BH3-only" that promote apoptosis, and its promoter region combines with HIF-1 to exert transcription-related functions. Moreover, Althaus et al. [29] showed that HIF-1α expression can induce an up-regulation of BNIP3 and promote apoptosis in a rat model of focal ischemia. Similarly, suppression of BNIP3 by HIF-1α inhibitors can reduce neural apoptosis following SAH [6, 10].

PFOB belongs to the non-toxic group of PFCs, has high stability, and is efficient in its oxygen-carrying ability. PFOB particles can achieve a minimum size of about 90 nm after emulsification, making it easier for clearance by vascular endothelial cells, prolonging its presence intravascularly and greatly increasing its oxygen-carrying capacity and penetration. PFCs were initially used for their special physical and chemical properties, and it was eventually found that PFOB protects against antioxidant stress damage [30], has anti-inflammatory effects [31], and has thus become been widely used in the clinical setting. In the field of neuro-

surgery, PFCs have been used in the recovery of consciousness after craniocerebral trauma, as well as to promote protection of neurons after spinal cord injury and hemorrhagic infarction [32, 33]. However, the use of PFOB nanoparticles in the treatment of SAH is still in its infancy. Our team previously showed that intravenous tail injection of PFOB nanoparticles in a rat model of prechiasmatic cistern SAH relieves brain edema, reduces EBI-induced neural apoptosis, and has neuroprotective effects [15]. However, no study has investigated the neuroprotective mechanism underlying the effects of PFOB nanoparticles. In the current study using a rat model of SAH (endovascular perforation), we found that treatment with 5 g/kg and 10 g/kg of PFOB nanoparticles at 1 h post- endovascular perforation in a rat model of SAH significantly reduced brain edema, EB extravasation, and hippocampal CA1 area neuronal apoptosis. Moreover, nanoparticles administration improved neurobehavioral functional deficits at 24 h after SAH, compared to both the SAH and SAH + vehicle groups. Notably, the effect of 10 g/kg of PFOB is more obvious than 5 g/kg of PFOB. This study further confirmed that PFOB nanoparticles had a neuroprotective effect against EBI after SAH. We also reported that 24 h after SAH, immunohistochemistry and western blots revealed significantly higher expression of HIF-1α, VEGF, and BNIP3, in the SAH group compared to the sham-operated group. Treatment with PFOB nanoparticles significantly reduced the SAH-induced increase in HIF-1α, VEGF and BNIP3. Moreover, the suppressant effect of SAH + 10 g/kg of PFOB was more apparent than that of SAH + 5 g/kg of PFOB. These results suggest that PFOB nanoparticles reduced EBI after experimental subarachnoid hemorrhage, and that this was related to inhibition of HIF-1α, VEGF and BNIP3 protein expression.

Although it is clear that PFOB nanoparticles can inhibit HIF-1α expression, the specific mechanisms of action remains unknown. HIF-1α protein expression depends mainly on two factors. The first mechanism by which HIF-1α expression is regulated is via oxygen levels: When oxygen is available, HIF proline hydroxylase is activated, andhydroxylates HIF-1α. The hydroxyl group of HIF-1α combines with von Hippel-Lindau disease tumor suppressor protein (pVHL), which can be recognized by E3

ubiquitin protease and result in HIF-1α degradation by 26 s protease [34, 35]. Under low oxygen conditions, proline hydroxylase has decreased activity, does not recognize the hydroxyl group of HIF-1α, and prevents HIF-1α ubiquitin degradation; thus, levels of HIF-1α protein are increased [34, 36]. The second factor involved in mediating HIF-1α expression is oxygen independent transcriptional regulation. The activation of multiple signaling pathways in cells can activate the synthesis of HIF-1α, which increases HIF-1α mRNA transcription and improves the synthesis of HIF-1α. This signaling pathway includes gene-silk crack, the original amp-activated protein kinase (Ras-MAPK), phosphoinositide 3-kinase/AKT protein kinase B and mammalian target of rapamycin (PI3K-AKT-mTOR) [37, 38]. Here, we found that both HIF-1α protein expression and mRNA levels were increased during EBI after SAH. However, treatment with PFOB nanoparticles did not reduce the rise in mRNA levels. One effect that administration of PFOB nanoparticles did have was on the downstream target genes of *HIF-1α*, specifically decreasing *VEGF* and *BNIP3* mRNA. Thus, we speculated that increases in HIF-1α occur through two aspects of transcriptional and post-transcriptional control, but that PFOBs reduce HIF-1α by a post-transcriptional mechanism. Moreover, we specifically suspect that PFOB nanoparticles increase HIF-1α ubiquitin degradation, thereby inhibiting the HIF-1α protein.

It should be noted that PFOB nanoparticles can play a neuroprotective role during EBI after SAH, and that the mechanisms of action are not restricted to the inhibition of HIF-1α expression. Thus, in order to promote the clinical use of PFOB nanoparticles, further research on safe dosing of PFOB and theirunderlying mechanisms of action following SAH is necessary.

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Disclosure of conflict of interest

None.

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