Norrin Protected Blood–Brain Barrier Via Frizzled-4/β-Catenin Pathway After Subarachnoid Hemorrhage in Rats

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Background and Purpose—Norrin and its receptor Frizzled-4 have important roles in the blood–brain barrier development. This study is to investigate a potential role and mechanism ofNorrin/Frizzled-4 on protecting blood–brain barrier integrity after subarachnoid hemorrhage (SAH).

Methods—One hundred and seventy-eight male adult Sprague–Dawley rats were used. SAH model was induced by endovascular perforation. Frizzled-4 small interfering RNA was injected intracerebroventricularly 48 hours after SAH. Norrin was administrated intracerebroventricularly 3 hours after SAH. SAH grade, neurological scores, brain water content, Evans blue extravasation, western blots, and immunofluorescence were used to study the mechanisms of Norrin and its receptor regulation protein TSPAN12, as well as neurological outcome.

Results—Endogenous Norrin and TSPAN12 expression were increased after SAH, and Norrin was colocalized with astrocytes marker glial fibrillary acidic protein in cortex. Exogenous Norrin treatment significantly alleviated neurobehavioral dysfunction, reduced brain water content and Evans blue extravasation, promoted β-catenin nuclear translocation, and increased Occludin, VE-Cadherin, and ZO-1 expressions. These effects were abolished by Frizzled-4 small interfering RNA pretreated before SAH.

Conclusions—Norrin protected blood–brain barrier integrity and improved neurological outcome after SAH, and the action of Norrin appeared mediated by Frizzled-4 receptor activation, which promoted β-catenin nuclear translocation, which then enhanced Occludin, VE-Cadherin, and ZO-1 expression. Norrin might have potential to protect blood–brain barrier after SAH. (Stroke. 2015;46:529-536. DOI: 10.1161/STROKEAHA.114.007265.)

Key Words: blood–brain barrier ▪ Frizzled-4 protein ▪ Norrin ▪ subarachnoid hemorrhage

The pathophysiology of subarachnoid hemorrhage (SAH) and other stroke events involve the cerebral vascular neural network, which includes arterial and venous systems as well as neuronal cells and other support cells and matrix. Within this vascular neural network, blood–brain barrier (BBB) damage contributes to brain edema after SAH. Therefore, to improve clinical outcomes of patients with SAH, it may be important to develop new therapies against BBB disruption.

Norrin, encoded by Norrie Disease Protein gene, is a secreted small molecule protein, which highly expressed in embryo development to regulate angiogenesis. Recent studies demonstrated that Norrin is also essential to BBB formation. Norrin activates the Frizzled-4 receptor, which is also the receptor for canonical Wnt/β-catenin pathway. The canonical Wnt/β-catenin pathway is reported to exhibit neuroprotective and angiogenesis actions.

However, it is unclear whether endogenous Norrin is induced and whether Norrin/Frizzled-4 will be protective against BBB disruption after SAH. This study investigated a potential role and mechanisms of Norrin and its receptor in the protection of BBB in a rat model of SAH (Figure I in the online-only Data Supplement).

Materials and Methods

Animals
One hundred and seventy-eight male adult Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 300 to 350 g were used in this study. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Experimental Design
The experiment was designed as follows (Figure II in the online-only Data Supplement).

Experiment 1
To determine the time course of Norrin and TSPAN12 after SAH, 20 rats were randomly assigned into 5 groups, such as sham (n=4),...
SAH 6 hours (n=4), SAH 12 hours (n=4), SAH 24 hours (n=4), and SAH 72 hours (n=4). Western blots were used to detect the protein expression of Norrin and TSPAN12 in ipsilateral/left hemisphere of each group. Double immunohistochemistry staining of Norrin and glial fibrillary acidic protein were also performed in 12 hours after SAH (n=1).

**Experiment II**

For outcome evaluation, 64 rats were randomly divided into 6 groups, such as sham (n=13), SAH (n=7), SAH+Vehicle (5-μL sterile saline; n=3), SAH+5 ng/μL r-Norrin (25 ng in 5-μL sterile saline; n=8), SAH+25 ng/μL r-Norrin (125 ng in 5-μL sterile saline; n=7) and SAH+50 ng/μL r-Norrin (250 ng in 5-μL sterile saline; n=14). Vehicle or r-Norrin was injected intracerebroventricularly 3 hours after SAH onset. Neurological Scores, including modified Garcia test and beam balance test, SAH grading scores and brain water content were assessed at 24 and 72 hours after SAH in all groups (n=6). Evans blue extravasation was evaluated at 24 hours after SAH in sham, SAH+Vehicle and SAH+50 ng/μL r-Norrin groups (n=5). Double immunohistochemistry staining of ZO-1 and von Willebrand factor (vWF) were also performed in sham, SAH, and SAH+50 ng/μL r-Norrin groups (n=2).

**Experiment III**

Nine rats were randomly assigned into 3 groups, such as sham (n=3), SAH+500 pmol scrambled small interfering RNA (siRNA; in 5-μL sterile saline; n=3), SAH+500 pmol Frizzled-4 siRNA (in 5-μL sterile saline; n=3). Scrambled siRNA or Frizzled-4 siRNA was intracerebroventricularly injected 48 hours before SAH.8 Western blots of ipsilateral/left hemisphere were conducted at 48 hours after injection in all groups.

Eighty rats were randomly divided into 5 groups for mechanism study: sham (n=10), SAH+Vehicle (n=11), SAH+50 ng/μL r-Norrin (250 ng in 5-μL sterile saline; n=11), SAH+50 ng/μL r-Norrin+500 pmol scrambled siRNA (in 5-μL sterile saline; n=24), and SAH+50 ng/μL r-Norrin+500 pmol Frizzled-4 siRNA (in 5-μL sterile saline; n=24). Scrambled siRNA or Frizzled-4 siRNA was intracerebroventricularly injected 48 hours before SAH modeling.10 Modified Garcia test and brain water content were performed at 24 hours after SAH in SAH+r-Norrin, Scrambled siRNA and SAH+r-Norrin+Frizzled-4 siRNA groups (n=6), so did Evans blue extravasation assessment (n=5). The data of these 3 tests in sham, SAH+Vehicle, and SAH+r-Norrin groups were shared with experiment II. Then, Evans blue fluorescence of ipsilateral/left cortex were also performed at 24 hours after SAH in sham, SAH, SAH+r-Norrin, and SAH+r-Norrin+Frizzled-4 siRNA groups (n=1). Western blots of ipsilateral/left hemisphere were conducted at 24 hours after SAH in all groups (n=10, 5 rats for total protein extraction, and other 5 rats for nucleic and cytoplasmic protein extractions).

**SAH Model**

SAH rat model was induced by endovascular perforation as previously described.11 All animals were transorally intubated after induced anesthesia with 5% isoflurane in 70:30 medical air:oxygen, and a small rodent respirator (Harvard Apparatus, Holliston, MA) was used to maintain an adequate respiration. Anesthesia was then maintained with 3% isoflurane in 70:30 medical air:oxygen. The external carotid artery was identified and transected distally with a 3-mm stump. A 4-0–sharpened monofilament nylon suture was advanced into the internal carotid artery through the external carotid artery until resistance was felt (at 18–20 mm) and then was pushed 5 mm further to penetrate the bifurcation of the anterior and middle cerebral artery. The suture was then withdrawn and the internal carotid artery was reperfused to produce SAH. Sham-operated rats underwent the same procedure except the suture was withdrawn without perforation after feeling resistance.

**Intracerebroventricular Injection**

Intracerebroventricular injection procedure was performed as reported previously.12 A small burr hole was drilled on the skull according to the following coordinates relative to bregma: 1.5 mm posterior and 1.0 mm lateral. The needle of 10-μL Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV) was stereotactically inserted into the left lateral ventricle through the burr hole 4.0 mm below the horizontal plane of bregma. Five microliters of r-Norrin (R&D system, Minneapolis, MN) in sterile saline were infused at a rate of 0.5 μL/min 3 hours after SAH induction, whereas 500 pmol/5 μL Frizzled-4 or scrambled siRNA (Invitrogen, Grand Island, NY) were infused at the same rate at 48 hours before SAH modeling. Frizzled-4 siRNA is a pool of 3 different siRNA duplexes to improve the knockdown efficiency. All Frizzled-4 siRNA sequences are provided in 5′→3′ orientation:

(I) Sense: CCG UUC UCA UCC AAG AGG GAC UUA A
Antisense: UUA AGU CCC UCU UGG AUG AGA ACG G

(II) Sense: GGCC ACU CUU UCG GUU UUC UGA AGA U
Antisense: AUC UGC AGA AUA CCG AAA GAG UGC C

(III) Sense: CCU AUU UGG UGA UUG GAA CUC UAU U
Antisense: AAU AGU GGU CCA AUC ACC AAA UAG G

**Figure 1.** Time course of endogenous Norrin and TSPAN12 expression after subarachnoid hemorrhage (SAH). A, Representative western blot bands of Norrin and TSPAN12 time course from ipsilateral hemisphere after SAH. Quantitative analyses of (B) Norrin and (C) TSPAN12 time course from ipsilateral hemisphere after SAH. D, Representative immunohistochemistry staining slices of Norrin and glial fibrillary acidic protein (GFAP) at 12 hours after SAH. Relative densities of each protein have been normalized against the sham group. n=4 #; vs sham P<0.05.
The syringe was left in situ for an additional 10 minutes before slowly removing. In experiment I, the sham group rats were subjected to the same procedure without inserting the needle.

Neurological Outcome Assessment
Neurological deficits were evaluated at 24 and 72 hours after SAH using an 18-point score system named Modified Garcia Scale and another 4-point score system named beam balance test in the outcome study.13 Modified Garcia assessment consisted of 6 tests covering spontaneous activity, spontaneous movement of 4 limbs, forepaw outstretching, climbing, body proprioception, and response to whisker stimulation (3–18 points). For beam balance test, the rats were placed on a beam to be observed their walking distance within 1 minute (0–4 points). The mean of neurological score was evaluated by 2 blinded observers for grading.

SAH Grade Assessment
An 18-point SAH severity grading system was used as previously described.14 The basal cistern was divided into 6 segments that can be scored from 0 to 3 according to the amount of subarachnoid blood clot. A total score was calculated by adding the scores from 6 segments (0–18 points). Animals received a score <8 should be excluded from the study.

Brain Water Content
The brains were quickly separated into the left and right cerebral hemispheres, cerebellum, and brain stem and weighed (wet weight). Then, the brain samples were dried in an oven at 105°C for 72 hours and weighed again (dry weight). The percentage of the water content was calculated as ([wet weight − dry weight]/wet weight)×100%.8

Evans Blue Extravasation and Fluorescence
Evans Blue extravasation was performed as reported previously.15 At 24 hours postoperation, the Evans blue dye (2%, 5 mL/kg; Sigma–Aldrich, St. Louis, MO) was injected and administered >2 minutes into the left femoral vein, where it was allowed to circulate for 60 minutes. Under anesthesia, the rats were euthanized by an intracardial perfusion with phosphate-buffered solution (PBS). After that the brains were removed and divided into the left and right cerebral hemispheres for homogenate. The brain samples were weighed, homogenized in saline, and centrifuged at 15,000 g for 30 minutes. Next, an equal volume of trichloroacetic acid was added to the resultant supernatant. The samples were then incubated overnight at 4°C and centrifuged at 15,000 g for 30 minutes. The resultant supernatant was then spectrophotometrically quantified for the extravasated Evans blue dye at 615 nm.

For Evans blue fluorescence, PBS was replaced by 4% paraformaldehyde after PBS intracardiac perfusion. Then the brains were removed to be prepared for coronal brain sections (10 μm) as same as immunofluorescence staining. And, red autofluorescence of Evans blue was observed on the slides using excitation and emission filters for rhodamine fluorescence15 (Olympus OX51, Tokyo, Japan).

Western Blotting
Western blot was performed as reported previously.16 The protein extracted from left hemisphere (perforation side) was used for western blot analysis. Membrane proteins were extracted by NE-PER Nuclear and Cytoplasmatic Extraction Reagents (Pierce Biotechnology, Rockford, IL), following instruction manual. Equivalent total, nuclear, or cytoplasmic protein amounts (30 μg) were loaded in each lane of SDS-PAGE gels. After gel electrophoresis, protein was transferred onto a nitrocellulose membrane, which was then blocked by blocking buffer for 2 hours at room temperature. Following primary antibodies were diluted to incubate with the membrane under gentle agitation at 4°C overnight: anti-Norrin, anti-TSPAN12 (Abcam), anti-β-catenin, anti–ZO-1, anti-Occludin, and anti–VE-Cadherin (Santa Cruz Biotechnology, Santa Cruz, CA). β-actin was used as an internal control for normalization of protein loading. The images were captured using a gel documentation system and analyzed by ImageJ software.

Figure 2. Effects of exogenous Norrin treatment on neurobehavioral outcomes after subarachnoid hemorrhage (SAH). Modified Garcia test results of each group at (A) 24 and (B) 72 hours after SAH. Beam balance test results of each group at (C) 24 and (D) 72 hours after SAH. n=6 #: vs sham P<0.05, *: vs SAH P<0.05, &: vs SAH+Vehicle P<0.05.
loading control by using anti–β-actin primary antibody (Santa Cruz Biotechnology). Appropriate secondary antibodies were incubated with the nitrocellulose membrane for 2 hours at room temperature. Chemiluminescent detection was performed to identify the immune bands with the kit (ECL Plus; Amersham Bioscience, Arlington Heights, IL). Data were analyzed by densitometry with Quantity One 4.6.2 (Bio-Rad Laboratories, Berkeley, CA).

**Immunofluorescence Staining**

Immunofluorescence staining for brain was performed on fixed frozen section as previously described. Twelve hours or twenty-four hours after SAH, rats were deeply anesthetized and transcardially perfused with PBS and 10% formalin. Rats’ brains were rapidly isolated and postfixed in 10% formalin for 24 hours and then in 30% sucrose for 3 days. Coronal brain sections (10 μm) were obtained with the help of cryostat (Leica CM3050S-3-1-1, Bannockburn, IL) and permeabilized with 0.3% Triton X-100 in PBS for 30 minutes. Sections were blocked with 5% donkey serum for 1 hour and incubated at 4°C overnight with primary antibodies: anti–ZO-1 (Abcam) and anti-vWF (Millipore, Temecula, CA); anti-Norrin (Abcam), and anti-glia fibrillary acidic protein (Santa Cruz Biotechnology) followed by fluorescein isothiocyanate-conjugated and Texas Red-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) for 2 hours at room temperature. The colocalization of ZO-1 with the marker of endothelial cells vWF and the colocalization of Norrin with the marker of astrocytes glial fibrillary acidic protein were examined by fluorescent microscope (Olympus OX51, Tokyo, Japan).

**Statistical Analysis**

One-way ANOVA followed by Tukey multiple comparisons test was used for different groups’ comparison. χ² tests were used for behavior score analyses. Data were showed as mean±SD. P<0.05 was considered statistical difference.

**Results**

**Time Course of Endogenous Norrin and TSPAN12 Expression After SAH**

SAH blood clots were cleared from the basal cisterns as time passed, but significant hemorrhage remained 72 hours after SAH. Western blot analysis demonstrated a significant elevation of Norrin in ipsilateral hemisphere after SAH except at 24 hours, with 2 peaks at 12 hours and 72 hours after SAH (Figure 1A and 1B). TSPAN12 was significantly increased at all 4 time points after SAH (Figure 1A and 1C). Furthermore, double immunohistochemistry staining at the first peak of Norrin (12 hours) after SAH showed that Norrin expression was colocalized with glial fibrillary acidic protein (Figure 1D) in left/ipsilateral cortex.

**Exogenous Recombinant Norrin (r-Norrin) Treatment**

Comparisons of SAH grading score revealed no significant differences among the groups either at 24 or 72 hours after SAH (Figure IIIA and IIIB in the online-only Data Supplement). None of the sham-operated rats died, and 6 rats died within 24 hours after SAH caused by severe hemorrhagic volume and 1 rat died during intracerebralventricular injection.

Compared with sham group, the rats from the SAH-, Vehicle- and r-Norrin 5 ng/μL groups showed significant neurological impairment on modified Garcia test both at 24 and 72 hours after SAH, but not after r-Norrin 25 ng/μL and 50 ng/μL treatments (Figure 2A and 2B). Further analysis
showed that both r-Norrin 25 and 50 ng/μL treatment significantly improved neurobehavioral outcomes 72 hours after SAH (Figure 2B), whereas only 50 ng/μL treatment was effective at 24 hours after SAH when compared with the sham or vehicle groups (Figure 2A).

For beam balance test, the rats from both SAH and vehicle groups also showed significant neurobehavioral dysfunction when compared with sham group, but not any of the r-Norrin treatment groups (Figure 2C and 2D). Only r-Norrin 50 ng/μL treatment significantly alleviated neurological impairment at 72 hours after SAH (Figure 2D).

Both SAH and vehicle groups showed increased brain water content at 24 hours after SAH in both hemispheres at 24 hours (Figure 3A) and in ipsilateral hemisphere at 72 hours after SAH (Figure 3B). r-Norrin 50 ng/μL treatment significantly reduced brain water content whereas 25 ng/μL treatment only reduced brain water content at contralateral hemisphere at 24 hours and ipsilateral at 72 hours after SAH (Figure 3A and 3B). Overall, high dosage of r-Norrin (50 ng/μL) was the most effective treatment and it was used for the rest of the experiments.

We used Evans blue extravasation to evaluate the BBB integrity after SAH. The results showed that there was more Evans blue dye leaking out of vessel in both hemispheres at 24 hours after SAH. The r-Norrin treatment significantly reduced Evans blue leakage in ipsilateral hemisphere (Figure 3C). Although there was no significant difference between SAH and r-Norrin treatment groups in contralateral hemisphere, no significant difference between sham and r-Norrin treatment groups were identified either, indicating r-Norrin effectively reduced Evans blue dye leakage (Figure 3C). In immunohistochemical staining, continuous endothelial cells (vWF) and ZO-1 structures broke up at 24 hours after SAH, and r-Norrin treatment effectively reduced those damages (Figure 3D).

### Specific Inhibition of Frizzled-4 Receptor Expressions Before Norrin Treatment

Comparisons of SAH grading score revealed no significant differences among the groups at 24 hours after SAH (Figure IIIC in the online-only Data Supplement). None of the sham-operated rats died, 6 rats died after SAH modeling, and 2 rats died after intracerebral ventricularly injection.

Frizzled-4 expression was measured by western blot and no difference was observed in scrambled siRNA pretreatment group when compared with sham, but Frizzled-4 siRNA pre-treatment significantly inhibited Frizzled-4 receptor expression in ipsilateral hemisphere at 48 hours after siRNA injection (Figure IVA and IVB in the online-only Data Supplement).

Frizzled-4 siRNA pretreatment sufficiently abolished the protective effect of r-Norrin as shown in modified Garcia test (Figure 4A), brain water content (Figure 4B), Evans blue extravasation (Figure 4C), and Evans blue fluorescence in left cortex (Figure 4D) when compared with r-Norrin treatment group.

### Expressions of Endothelial Junction Proteins and β-Catenin After Treatment

The expression of Occludin, VE-Cadherin, and ZO-1 was significantly reduced at 24 hours after SAH (Figure 5A–5D),
whereas nucleic β-catenin levels increased (Figure 6A and 6D) even though the total and cytoplasmic levels remained (Figure 6A–6C). r-Norrin preserved the expression levels of

Figure 5. Changes of endothelial junction proteins expression after treatment at 24 hours post subarachnoid hemorrhage (SAH). Representative bands (A) and quantitative analysis of Occludin (B), VE-Cadherin (C), and ZO-1 (D) expressions in ipsilateral hemisphere of brain specimen at 24 hours after SAH. Relative densities of each protein have been normalized against the sham group. Fzd4 indicates Frizzled-4; Scr, scrambled; and siRNA, small interfering RNA. n=5 #: vs sham P<0.05, *: vs SAH+Vehicle P<0.05, and &: vs SAH+Scr siRNA P<0.05.

Figure 6. Changes of β-catenin expression after treatment at 24 hours post subarachnoid hemorrhage (SAH). Representative bands (A) and quantitative analysis of β-catenin expression in total (B), cytoplasmic (C), and nucleic (D) proteins in ipsilateral hemisphere of brain specimen at 24 hours after SAH. Relative densities of each protein have been normalized against the sham group. Fzd4 indicates Frizzled-4; Scr, scrambled; and siRNA, small interfering RNA. n=5 #: vs sham P<0.05, *: vs SAH+Vehicle P<0.05, and &: vs SAH+Scr siRNA P<0.05.
Occludin, VE-Cadherin, and ZO-1, and increased the nuclear portion of β-catenin levels when compared with sham and vehicle groups (Figures 5A–5D and 6A–6D).

Frizzled-4 siRNA pretreatment decreased the expression levels of Occludin, VE-Cadherin, and ZO-1 compared with r-Norrin treatment group, whereas scrambled siRNA did not show those effects (Figure 5A–5D). Frizzled-4 siRNA pretreatment but not scrambled siRNA decreased the nuclear β-catenin levels when compared with SAH+r-Norrin group (Figure 6A and 6D).

Discussion

This study demonstrated that endogenous Norrin was increased in brain tissues with 2 peaks at 12 and 72 hours post-SAH. The essential Norrin supporting protein TSPAN12 was also increased ≤ 72 hours after SAH. Recombinant Norrin protein alleviated neurological impairment and BBB disruption, which were associated with β-catenin translocation from cytoplasmic into nuclear and an increase of Occludin, VE-Cadherin, and ZO-1 protein expressions. Furthermore, blockade of Frizzled-4 receptor by siRNA at 48 hours before SAH eliminated these protective effects of Norrin, prevented β-catenin translocation, and reduced those 3 endothelial junction proteins, such as Occludin, VE-Cadherin, and ZO-1 expressions. Taking together, these observations suggested that Norrin may be a protective factor against BBB disruption after SAH.

Previous studies suggested that Norrin is essential to BBB development and maintenance, even though its underlying mechanisms are unclear. Endothelial tight junction proteins, such as Occludin and ZO-1, and endothelial adherent proteins like VE-Cadherin, are important to BBB integrity. It has been suggested that canonical β-catenin translocation binds to T-cell factor/lymphoid enhancer factor and initiates downstream protein transcription. The only reported receptor of Norrin is Frizzled-4, which happens to be the same receptor of canonical β-catenin pathway. In addition, this pathway has been suggested to promote angiogenesis and protect BBB. The observations of this study are consistent with previous reports that both neurological dysfunction and BBB disruption were reduced by exogenous recombinant Norrin, whereas the action of Norrin was eliminated by inhibition of Frizzled-4 receptor. This observation indicated that the protective effect of Norrin may be mediated by Frizzled-4 receptor, which in turn promoted β-catenin nuclear translocation, enhanced Occludin, ZO-1, and VE-Cadherin transcription and expression.

Norrin is highly expressed in embryo development, but barely detected in adult rodent animals. In addition, Norrin was thought to be expressed only in cerebellum and retina in central nervous system. This might be caused by a regulate protein TSPAN12, which could specifically enhance Norrin/Frizzled-4 pathway, but not Wnt/Frizzled pathways in brain tissues after SAH. Wnt protein is widely expressed and nonspecifically affected, which is difficult to manipulate and could cause high bone mass, even cancer, if Wnt is enhanced; or osteoporosis and heart failure if Wnt is inhibited. However, Norrin is a autocrine/paracrine protein with low abundance in normal adults, which has local effect toward surrounding cells. An increase of TSPAN12 in central nervous system could prominently enhance the protective effects of Norrin but not Wnt. Furthermore, the molecular weight of Norrin is relatively low, which may has more potential BBB permeability after peripheral administration and easy to be delivered into central nervous system, but pharmaceutical and pharmacokinetic studies of Norrin treatment are still needed in the future studies.

This study has limitations that it is focused on the Norrin/Frizzled-4 pathways but not designed to study β-catenin translocation in details. β-catenin translocation, which enhances junction protein transcription was well established in other studies. In addition, Norrin was thought to be secreted by astrocyte, and affecting surrounding cells, which consist with this study. But it cannot be ruled out other unknown mechanisms of Norrin, thus in our future studies, other particular cell-location and mechanisms of Norrin are still needed to be clarified.

Conclusions

This study demonstrated for the first time that endogenous Norrin elevation occurred after SAH in brain tissues, and extraneous recombinant Norrin protected BBB and improved neurological outcome, mediated possibly by Frizzled-4, which may in turn promoted β-catenin nuclear translocation, and then enhanced junction protein expressions in the brain. Recombinant Norrin might be a promising treatment option for BBB protection after SAH.

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Disclosures

None.

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Supplementary Material

Supplemental Figure I: Proposed pathway in the present study
Supplemental Figure II: Experimental design and animal groups’ classification. 

WB = Western blot; EB = Evans blue extravasation; IHC = immunohistochemistry; Scr = Scrambled; Fzd4 = Frizzled 4; ICV = intracerebralventricular.
Supplemental Figure III: SAH grading of each group after SAH

SAH grading scores of each group in Experiment I at 24h(A) and 72h(B) after SAH and in Experiment II at 24h after SAH. Scr = Scrambled; Fzd4 = Frizzled 4.
Supplemental Figure IV: Inhibition effect of Frizzled siRNA

Representative bands(A) and quantitative analysis(B) of the Frizzled siRNA inhibiting effect. Relative densities of each protein have been normalized against the sham group. Scr = Scrambled; Fzd4 = Frizzled 4. n=5  &: vs. SAH+Scr siRNA  P<0.05