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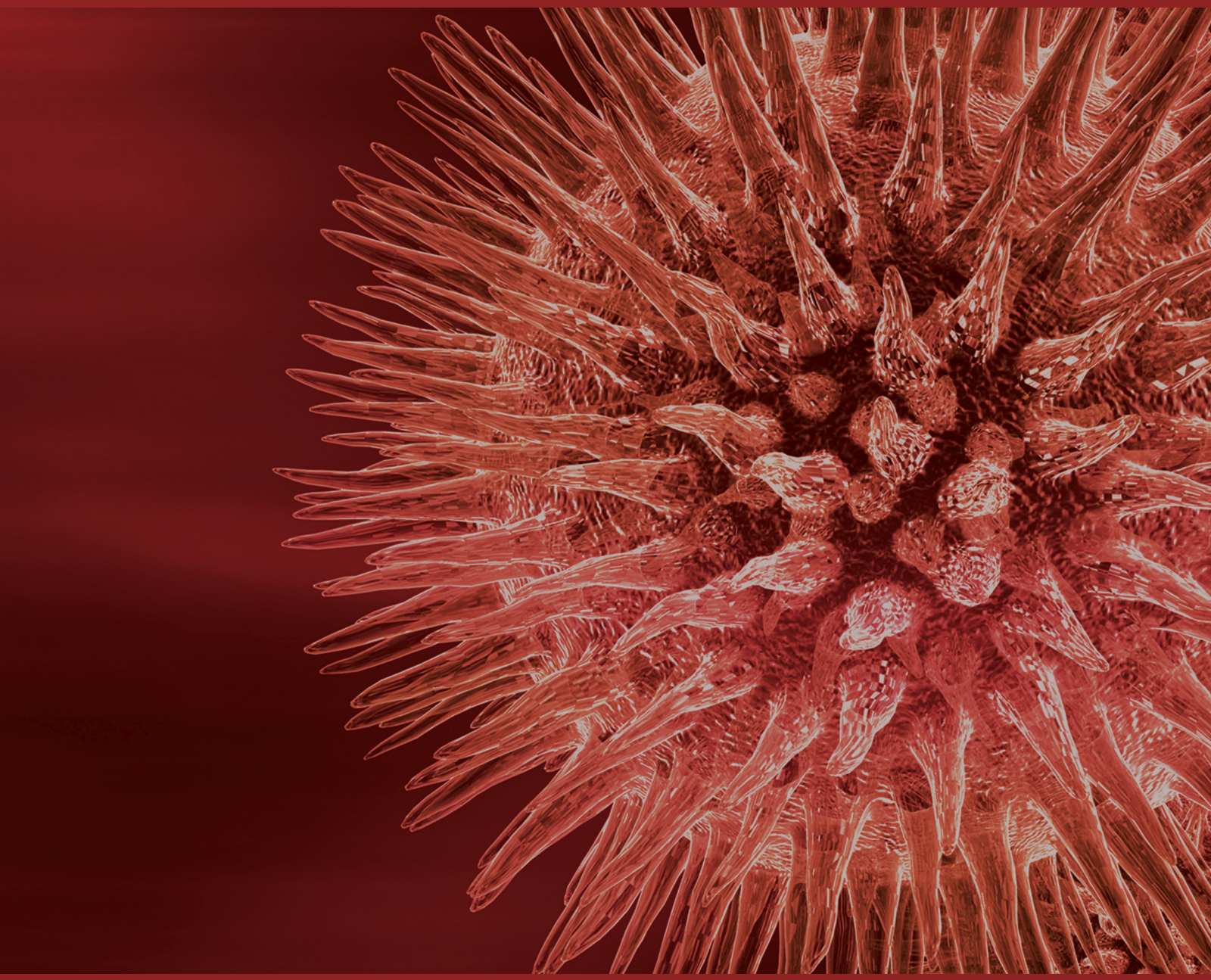
Guest Editors: Chih-Lung Lin, Aaron S. Dumont, John H. Zhang, Mario Zuccarello, and Carl Muroi



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Editorial

Cerebral Vasospasm after Aneurysmal Subarachnoid Hemorrhage: Mechanism and Therapies

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Although cerebral vasospasm (CV) after aneurysmal subarachnoid hemorrhage (SAH) has been recognized for more than half a century, its pathophysiologic mechanism remains elusive [1]. Delayed CV has classically been considered as the leading and treatable cause of mortality and morbidity in patients following aneurysmal SAH. Despite intensive research efforts, SAH-induced CV remains incompletely understood from both the pathogenic and the therapeutic perspectives. Many pathological processes have been proposed to explain the pathogenesis of delayed CV after SAH, including endothelial damage, smooth muscle contraction, changing in vascular responsiveness, and inflammatory and/or immunological response of the vascular wall [2]. At present, the most important and critical aspects of SAH-induced CV are its failure to consistently respond to treatment and only partial success could be achieved in both experimental models and clinical trials.

For patients with SAH surviving the early phase, secondary ischemia (or delayed cerebral ischemia, DCI) is popularly considered as the leading determinant of poor clinical outcome. Amongst the complications after SAH, CV has been regarded as the major cause of DCI. However, there have been an increasing number of evidences supporting multiple etiologies of DCI other than CV. Although radiographic CV is presented in up to 70% of SAH patients, only 20–30% of all SAH patients suffer from clinically symptomatic CV [3]. Nonetheless, it is now evident that CV alone is inadequate to completely explain DCI following aneurismal rupture [2, 4]. Recent studies on the treatment of CV have failed to solidly support the correlation between angiogram-shown improvement in CV and prognosis. Besides, various drugs proven effective for better functional outcomes have demonstrated their independency of CV reduction. Currently, a multifactorial etiology for DCI has emerged, whereas the role of CV has shifted from the major and most significant determinant to one contributing factor, just like any other factors, to the process. The study of the pathophysiology of DCI has become more broad-minded with several other different mechanisms being actively investigated.

The term "early brain injury" (EBI) was first postulated in 2004, more than 40 years after delayed CV was first described, to explain the acute pathophysiological events occurring within 72 hours of SAH [5, 6]. These events include cerebral autoregulation and blood-brain barrier disruption, activation of inflammatory pathways, excitotoxicity, oxidative stress, and activation of apoptosis [7]. These are direct effects of blood clot in the subarachnoid space and also of transient cerebral ischemia, leading to brain injury not confined to

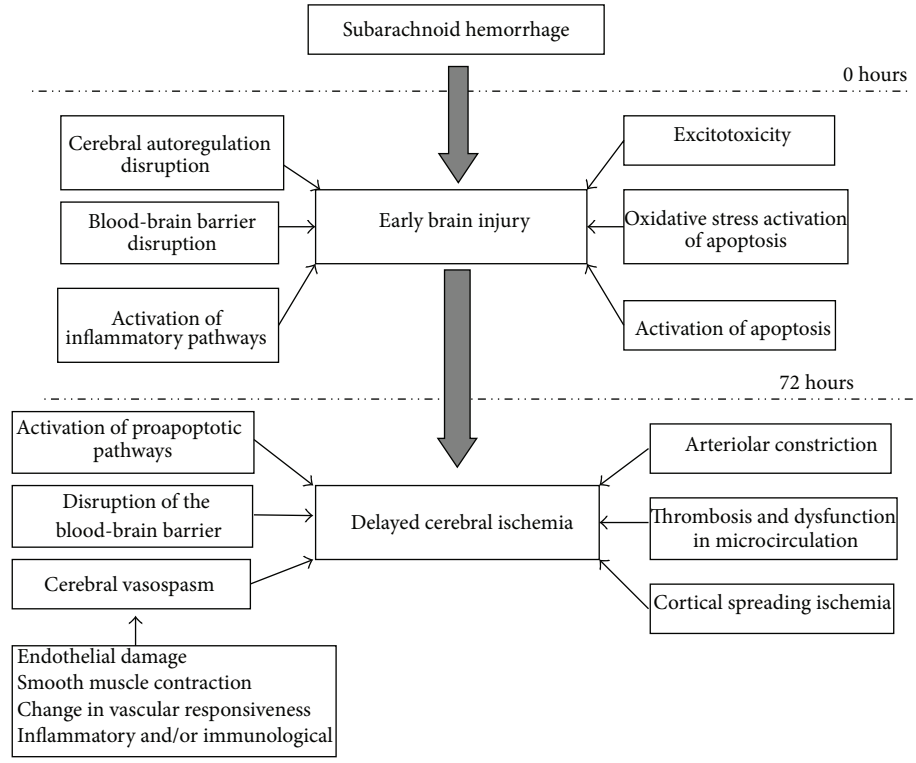

Figure 1: The mechanisms of early brain injury and delayed cerebral ischemia following subarachnoid hemorrhage.

the site of hemorrhage. Many mechanisms of EBI contribute to the pathogenesis of DCI and are hence accountable for the poor outcomes. Causes of DCI have been attributed to the combined effects of delayed CV, activation of proapoptotic pathways, disruption of the blood-brain barrier, arteriolar constriction, thrombosis and dysfunction in microcirculation, and cortical spreading ischemia, all brought about by EBI [2].

Accumulating data have suggested that apoptosis is a key mediator of secondary brain injury after SAH [8]. Approximately, 50% of SAH survivors remain permanently disabled because of cognitive dysfunction and do not return to their previous functions [9]. CV alone could not explain the whole subtle changes in behavior and memory. In this aspect, apoptosis induced by global ischemia should be taken into consideration.

In this special issue, an update review of the mechanism and treatment of CV and DCI after aneurysmal SAH is presented. The roles of mechanisms including microclot formation, downregulation of endothelial nitric oxide synthase, and upregulation of relaxin are discussed. Treatment with progesterone, which attenuates experimental SAH-induced CV by upregulation of endothelial nitric oxide synthase via Akt signaling pathway, is investigated. Besides, a study on Magnesium Lithospermate B, an active extract of salvia miltiorrhiza mediating sGC/cGMP/PKG translocation to reduce CV, is reported. Furthermore, new strategies using 17β-estradiol, targeting at several CV-preventing mechanisms, have brought light to the reduction of CV and secondary brain injury after SAH. The treatment and outcome including extracerebral organs damage and long-term complications after aneurysmal and nonaneurysmal SAH are also presented. Medical resources utilization in patients following SAH between the medical center and regional hospital is reported on a nationwide population-based study.

DCI, a result of different pathological pathways, is a complex process and has shown its importance as the leading determinant of poor functional outcome in patients surviving the initial hemorrhagic insult of SAH. The possible mechanisms of EBI and DCI after SAH, as well as their relationship with CV, are illustrated in Figure 1. The importance of CV in DCI has long been overemphasized. CV is not the sole or necessary process leading to DCI. Treatment strategies targeting at CV prevention alone are not adequate. Considering CV as the only monitor of therapeutic effectiveness or the lone prognostic marker can be misleading. Strategies focusing on the detection and treatment of EBI as an alleviation of the occurrence of DCI to subsequently improve overall outcome could make promising future study blueprints.

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Research Article

Upregulation of Relaxin after Experimental Subarachnoid Hemorrhage in Rabbits

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Background. Although relaxin causes vasodilatation in systemic arteries, little is known about its role in cerebral arteries. We investigated the expression and role of relaxin in basilar arteries after subarachnoid hemorrhage (SAH) in rabbits. *Methods*. Microarray analysis with rabbit basilar artery RNA was performed. Messenger RNA expression of relaxin-1 and relaxin/insulin-like family peptide receptor 1 (RXFP1) was investigated with quantitative RT-PCR. RXFP1 expression in the basilar artery was investigated with immunohistochemistry. Relaxin concentrations in cerebrospinal fluid (CSF) and serum were investigated with an enzyme-linked immunosorbent assay. Using human brain vascular smooth muscle cells (HBVSMC) preincubated with relaxin, myosin light chain phosphorylation (MLC) was investigated with immunoblotting after endothelin-1 stimulation. *Results*. After SAH, RXFP1 mRNA and protein were significantly downregulated on day 3, whereas relaxin-1 mRNA was significantly upregulated on day 7. The relaxin concentration in CSF was significantly elevated on days 5 and 7. Pretreatment with relaxin reduced sustained MLC phosphorylation induced by endothelin-1 in HBVSMC. *Conclusion*. Upregulation of relaxin and downregulation of RXFP1 after SAH may participate in development of cerebral vasospasm. Downregulation of RXFP1 may induce a functional decrease in relaxin activity during vasospasm. Understanding the role of relaxin may provide further insight into the mechanisms of cerebral vasospasm.

1. Introduction

Cerebral vasospasm is one of the most important cerebrovascular events following subarachnoid hemorrhage (SAH) and is characterized by delayed and prolonged contraction of cerebral arteries that may cause cerebral ischemia and lead to death or neurological deficits in patients with SAH [1]. Therefore, the prevention as well as treatment of vasospasm is important in the management of SAH patients. Although increased production of spasmogens and increased vascular responsiveness can be attributed to cerebral vasospasm, the mechanism of cerebral vasospasm remains elusive, and thus effective therapeutic strategies are not available. Recent randomized clinical trials have shown that currently available antivasospastic drugs are not sufficient to improve outcome [2]. Therefore, further research efforts are needed to clarify the mechanism of vasospasm and find new therapeutic targets.

Relaxin is a small peptide hormone (6 kDa) that is primarily produced by the corpus luteum, decidua, and placenta during pregnancy [3]. Three relaxin genes have been identified in humans and are designated as relaxin-1 (*RLN1*), relaxin-2 (*RLN2*), and relaxin-3 (*RLN3*). Human relaxin-2 is the only form of circulating relaxin that is substantially increased during pregnancy [4]. Human relaxin-2 is functionally equivalent to relaxin-1 in all other mammals [5]. Recently, *RLN* mRNA expression has also been detected in nonreproductive tissues including arteries, heart, kidney, liver, and lung [6–8].

Four relaxin receptor genes have been identified. They are relaxin/insulin-like family peptide receptors and are named RXFP1 (*RXFP1*), RXFP2 (*RXFP2*), RXFP3 (*RXFP3*), and

RXFP4 (*RXFP4*) [8]. Circulating relaxin (relaxin-2 in humans and relaxin-1 in other mammals) binds to *PRXFP1* (previously known as leucine-rich repeat-containing G-protein-coupled receptor 7: LGR 7) with high affinity [7, 9, 10]. RXFP1 mRNA and protein are expressed in a wide range of reproductive tissues including ovary, uterus, placenta, mammary gland, prostate, and testis [8]. The receptor is also expressed in nonreproductive tissues including heart, kidney, lung, liver, and vasculature [8]. Beyond a role in the reproductive system during pregnancy, a growing body of literature suggests that relaxin has extensive cardiovascular effects such as promoting vasodilation and angiogenesis and protecting against fibrosis and inflammation in systemic and renal circulation [11, 12].

Several recent studies have reported that relaxin and RXFP1 (LGR7) are expressed in the local arteries of mice and rats [13, 14]. These molecules are localized in the local arterial wall and seem to contribute to increased arterial compliance and reduced myogenic reactivity, and they may mediate blood flow to tissues [13, 14].

Based on the potent cardiovascular effects of relaxin, we hypothesize that relaxin dilates the cerebral arteries and plays a role in mediating cerebral blood flow. To date, however, no study has explored the expression and role of relaxin and its receptor, RXFP1, in the cerebral arteries after SAH. Therefore, the purpose of the present study was to investigate the time course of *RLN* and *RXFP1* expression in the cerebral arteries after SAH and to clarify the role of relaxin during vasospasm.

2. Materials and Methods

2.1. Preparation of the Rabbit SAH Model. This study was performed in accordance with the guidelines for proper conduct of animal experiments published by the Science Council of Japan. The study protocol was approved by the Animal Care and Use Committee, Kyushu University (Permit number A24-103-0). Adult male Japanese white rabbits (2.5 to 3.0 kg) were anesthetized with an intramuscular injection of ketamine (40 mg/kg body weight) and given an intravenous injection of sodium pentobarbital (20 mg/kg body weight). On day 0, 0.5 mL cerebrospinal fluid (CSF) was aspirated percutaneously from the cisterna magna using a 23-gauge butterfly needle, and then 2.5 mL nonheparinized autologous arterial blood that was obtained from the central ear artery was injected into the cisterna magna over 1 minute. The animal was kept in a prone position with the head tilted down at 30° for 30 minutes. During this procedure, no blood clot formation was observed in the syringe. On day 2, a similar second injection of autologous blood was performed. In this study, rabbits that did not undergo any surgical procedures including puncturing of skin or dura mater with a needle were used as control model (day 0). One of the reasons why nonmanipulated rabbit was used as the control model is to curb the number of rabbits used as possible from the point of view of animal ethics.

2.2. Harvest of Rabbit Basilar Artery. On days 0, 3, 5, and 7 after the first hemorrhage, the rabbits were heparinized (400 U/kg body weight), euthanized by intravenous injection of an overdose of sodium pentobarbital (120 mg/kg body weight), and exsanguinated from the common carotid artery. Exposure of the brain revealed clot formation over the surface of the pons and the basilar artery in the SAH animals. Immediately after removing the whole brain *en bloc*, the subarachnoid membrane was carefully dissected, and the clot was gently removed under a binocular microscope (Leica EZ4D, Leica Microsystems, Wetzler, Germany) with microscissors and microforceps so as not to touch the basilar artery. The distal half of the surface of the basilar artery in particular was covered with a thick clot in all rabbits with SAH. To estimate vasospasm, the external diameter of the basilar artery was measured at a location that was one-third the length from the distal end of the basilar artery. The ventral surface of the whole brain was photographed with a digital camera (CX3, Richo, Tokyo, Japan), and the external diameter of the basilar artery was analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA). The entire length of the basilar artery was then immediately excised from the brain and dissected free from surrounding tissues with microscissors and microforceps. Intraluminal blood was gently hand-flushed out with normal physiological salt solution (123 mmol/L NaCl, 4.7 mmol/L KCl, 1.25 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 1.2 mmol/L KH_2PO_4, 15.5 mmol/L $NaHCO_3$, and 11.5 mmol/L d-glucose) using a tuberculin syringe, and then the basilar artery was frozen in liquid nitrogen and stored at −80°C until use.

2.3. Total RNA Isolation from the Rabbit Basilar Artery. Total RNA was extracted from the basilar artery using the TRIZOL Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocols. The quality of total RNA was evaluated with a spectrophotometer (Nano-Drop2000c; Thermoscientific, Wilmington, DE) and gel electrophoresis (Experion; Bio-Rad, Hercules, CA, USA). RNA samples with an A260/280 ratio higher than 1.8 were used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. In addition to evaluation with a spectrophotometer, RNA samples with an RNA Quality Index higher than 9.0 were used for gene expression microarray analysis.

2.4. Gene Expression Microarray Analysis of the Rabbit Basilar Artery. Total RNAs extracted from rabbit basilar arteries on days 0, 3, 5, and 7 after the first hemorrhage ($n = 3$ each) were used for the microarray analysis. From 50 ng total RNA, cRNA was amplified, labeled, and hybridized to a rabbit gene expression microarray (Agilent Technologies, Santa Clara, CA, USA) using the Low Input Quick Amp one-color Labeling kit (Agilent Technologies) according to the manufacturer's instructions. All hybridized microarray slides were scanned with an Agilent Microarray scanner G2505B (Agilent Technologies). Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1) (Agilent Technologies). According to the manufacturer's instructions, raw signal intensities and flags for each probe were calculated from hybridization intensities and spot information. The raw signal intensities of the samples were $\log_2$ transformed

and normalized using a quantile algorithm with the "preprocessCore" library package of Bioconductor software [15, 16]. Then, we identified differentially expressed genes in the SAH model using the linear models for microarray analysis (limma) package of Bioconductor software [16, 17]. Genes in SAH samples with a limma value of $P < 0.05$ and an absolute limma $\log_2$ fold change ($|\log_2$ fold change$|$) higher than 1.0 compared to the control samples were defined as differentially expressed genes in this study. Microarray data are available from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE44910.

2.5. Quantitative RT-PCR Analysis of mRNA Expression of RLN1 and RXFP1 in the Rabbit Basilar Artery. Total RNAs extracted from rabbit basilar arteries on days 0, 3, 5, and 7 after the first hemorrhage were used for RT-PCR ($n = 5$ each). Complementary DNA (cDNA) was synthesized at 42°C for 30 minutes using 200 ng RNA template in a 20 μL reaction mixture containing high capacity RNA-to-cDNA master mix (Applied Biosystems, Foster City, CA, USA) with an ABI 2720 thermal cycler (Applied Biosystems). The cDNA was stored at −80°C until use in qRT-PCR. qRT-PCR was performed in triplicate in a 20 μL reaction mixture containing TaqMan Fast Universal PCR master mix (Applied Biosystems), 10 ng cDNA, and components of the TaqMan gene expression assay kit (rabbit *RLN1*: Oc03398001_m1, Applied Biosystems) or Custom TaqMan gene expression assay kit (rabbit *RXFP1*: forward primer 5′-GCATTCTCCAGAGAGTGTTTGTCT-3′, reverse primer 5′-GGCGCATGCAGATGACAAAA-3′, TaqMan probe 5′-ACTGCGGAGACCACC-3′, Applied Biosystems) using an ABI 7500 Fast Real-Time PCR system thermal cycler (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (rabbit *GAPDH*: Oc03823402_g1) was amplified as an endogenous control. The PCR protocol was composed of initial denaturation at 95°C for 20 seconds, followed by 40 amplification cycles of 95°C for 3 seconds and 60°C for 30 seconds. The data were analyzed with the comparative cycle method. The relative amount (X_0/R_0) of target gene mRNA was calculated with the Ct value for the target gene mRNA (CtX) and the Ct value for GAPDH mRNA (CtR) in the same sample using the formula $X_0/R_0 = 2^{\mathrm{CtX}-\mathrm{CtR}}$, where X_0 is the original amount of target gene mRNA and R_0 is the original amount of GAPDH. The level of target gene expression on day 0 was assigned a value of 100%. The data were expressed as the mean values ± SEM.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis of Relaxin in Rabbit Cerebrospinal Fluid (CSF) and Serum. Rabbit CSF ($n = 6$) and serum ($n = 15$) samples were collected on days 0, 3, 5, and 7. After rabbits were anesthetized and positioned as described above, CSF (0.5 mL) and arterial blood (2 mL) were collected from the cisterna magna and central auricular artery, respectively. CSF samples were centrifuged at 10,000 rpm for 5 minutes at 4°C to remove red blood cells, nonactivated platelets, and other cellular debris. The supernatant was then frozen at −80°C for later analysis. Rabbit arterial blood (2 mL) was collected from the rabbit ear artery and transferred into anticoagulant-free vacuum tubes (Venoject VP-AS109 K50, Terumo Corporation, Tokyo, Japan). After centrifugation at 1,500 ×g for 15 minutes at 4°C, the serum was pipetted into tubes and frozen at −80°C for later analysis. For detection and quantification of the relaxin concentration in rabbit CSF and serum, a commercially available Relaxin ELISA kit (USCN Life Science, Wuhan, China) was used according to the manufacturer's instructions. Standards and all samples were measured in duplicate.

2.7. Perfusion Fixation of the Rabbit Basilar Artery. On days 0, 3, 5, and 7 ($n = 3$ each) after the first hemorrhage, rabbits were anesthetized as described above. A thoracotomy was performed, the left ventricle was cannulated with an 18 G catheter (Surflo IV Catheter, Terumo Corporation), the right atrium was opened widely, and the descending thoracic aorta was clamped. Perfusion was begun with 500 mL heparinized saline (10 U/mL), followed by 500 mL 4.0% paraformaldehyde through a cannula in the left ventricle with a perfusion pressure of 100 cmH_2O. The brain including the basilar artery was removed and stored in 4.0% paraformaldehyde solution at 4°C overnight. For histological examination, the brain including the basilar artery was embedded in paraffin and cut into 6 μm sections.

2.8. Immunohistochemical Staining Analysis of RXFP1 Expression in the Rabbit Basilar Artery. The sections were deparaffinized, and endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in methanol for 30 minutes at room temperature. Heat-induced epitope retrieval was performed at 120°C for 20 minutes in 0.01 M citrate buffer. The sections were then incubated with blocking serum (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) followed by incubation with monoclonal anti-mouse LGR7 (RXFP1) antibody (1 : 100, Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight. Sections were incubated with biotinylated horse anti-mouse antibodies and then incubated with avidin-horseradish peroxidase (HRP) conjugate (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's recommendations for the Vecstain Elite ABC kit. The staining procedure was performed using a Vectastain Elite ABC kit (Vector Laboratories) and 3,3′-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The sections were then counterstained with hematoxylin and observed under a microscope (BZ-8000, Keyence, Osaka, Japan). Quantitative analysis was performed by modification of previously described methods [18]. Immunoreactivity was quantitatively analyzed by placing a 25 × 25 μm square over the most intensely stained area in the tunica media, and densitometric analysis was performed with image analysis software (BZ-Analyzer, Keyence).

2.9. Cell Culture, Treatment, and Protein Extraction. Human brain vascular smooth muscle cells (HBVSMC) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in smooth muscle cell medium, which consists of 500 mL basal medium, 10 mL fetal bovine serum, 5 mL smooth muscle cell growth supplement, and 5 mL penicillin/streptomycin solution. Cell cultures were maintained at

37°C in a humidified atmosphere with 5% CO_2. Cells from passages 5 to 10 were used for the study.

For experiments, cells were seeded at 3–5 × 10^5 cells/6 cm dish and grown until 90% confluent. Then, cells were incubated in serum-free smooth muscle cell medium (ScienCell Research Laboratories) in the presence or absence of 10 nmol/L human recombinant relaxin-2 (H2-relaxin, Peprotech, Rocky Hill, NJ, USA) for 24 hours. After stimulation with 100 nmol/L endothelin-1 (Peptide Institute, Osaka, Japan) for 0, 5, 10, and 30 minutes with or without relaxin treatment, smooth muscle cell proteins were extracted as follows.

At the indicated time points, cells were rinsed three times with ice-cold phosphate-buffered saline (PBS: 136.9 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4 $12H_2O$, and 1.47 mmol/L KH_2PO_4) and lysed with ice-cold cell lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.5% (v/v) Nonidet P-40, and 5 μmol/L microcystin-LR). Immediately after scraping the cells off the dishes, cell lysates were snap-frozen in liquid nitrogen and thawed at room temperature. After centrifugation for 15 minutes at 12,000 rpm at 4°C, the supernatants were collected. The protein concentrations of the supernatants were measured using the Bradford method (Thermo Fisher Scientific, Waltham, MA, USA). The samples were diluted to a final concentration of 1 mg/mL with the cell lysis buffer and 4× LDS NuPAGE sample buffer (Invitrogen) and stored at −80°C until use.

2.10. Analysis of Myosin Light Chain (MLC) Phosphorylation with Phos-Tag Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Phosphorylation of MLC was analyzed using a new method based on Phos-tag technology. Phos-tag is a compound that specifically binds to phosphate groups. Therefore, SDS-PAGE containing polyacrylamide-bound Mn^{2+} Phos-tag (Phos-tag SDS-PAGE) causes a mobility shift, depending on the degree of phosphorylation [19].

Before electrophoresis, the samples were heated at 100°C for 5 minutes and equilibrated to room temperature. The 2 μg protein samples were separated on 12.5% (w/v) polyacrylamide gels containing 30 μM Phos-tag Acrylamide (NARD Institute, Hyogo, Japan) for SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). Electrophoresis was performed in 0.1% (w/v) SDS, 25 mmol/L Tris-hydroxymethyl aminomethane, and 192 mmol/L glycine at 12 mA constant current/8 cm × 5 cm × 0.75 mm gel for 155 minutes. After electrophoresis, the gel was soaked for 30 minutes in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, and 10% (v/v) methanol) containing 2 mmol/L EDTA to remove the Mn^{2+} and then in transfer buffer without EDTA for 15 minutes. Proteins were then transferred to polyvinylidene difluoride membranes (0.2 μm pore size; Bio-Rad) in transfer buffer for 2 hours at room temperature. The membranes were then washed in PBS for 5 minutes and treated with 0.5% (w/v) formaldehyde in PBS for 45 minutes. After a brief wash in PBS, the membrane was blocked with 5% (w/v) skimmed milk in T-TBS overnight at 4°C. All forms of 20 kDa MLCs were detected on the immunoblot using a rabbit polyclonal anti-MLC antibody (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1 : 1000; Sigma) diluted in immunoreaction enhancer solution (Can Get Signal; Toyobo, Osaka, Japan). The immune complex was detected using enhanced chemiluminescence (ECL plus kit; Amersham, Buckinghamshire, UK). The light emission was detected and analyzed with VersaDoc 5000 and the computer program Quantity One (Bio-Rad). The percent of phosphorylated MLC of the total MLC (sum of unphosphorylated and phosphorylated forms) was calculated to indicate the extent of MLC phosphorylation.

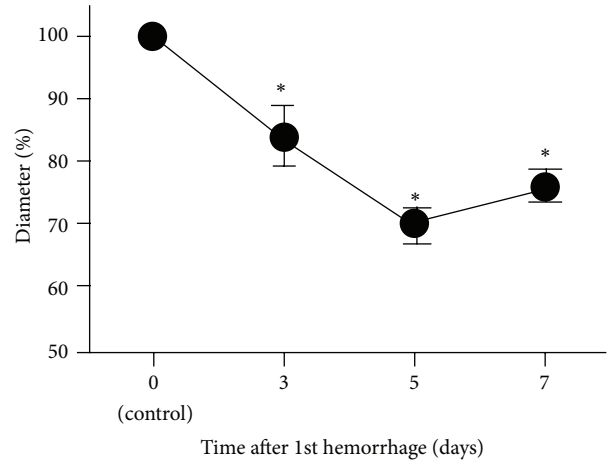

Figure 1: Time course of narrowing of the rabbit basilar artery after subarachnoid hemorrhage (SAH). Time course of changes in the external diameter of rabbit basilar arteries after SAH. Data are the means ± SEM (n = 3, each time point). The external diameter on day 0 was considered 100%. $^*P < 0.05$ versus day 0 (control).

2.11. Statistical Analysis. The data are expressed as the mean value ± SEM of the indicated experimental number. One basilar arterial preparation obtained from one animal was used for each experiment, and therefore the number of experiments (n value) equals the number of rabbits. An analysis of variance followed by Dunnett's post hoc test was used to determine statistically significant differences in a multiple comparison with the control model. A value of $P < 0.05$ was considered statistically significant. All analyses were performed using GraphPad PRISM software version 5.0 (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Assessment of Cerebral Vasospasm following SAH. The external diameter of the basilar artery was 0.87 ± 0.048 mm in the controls (day 0, before SAH induction). On day 3, the basilar artery was significantly narrowed to 84.0 ± 4.7% of the control ($P < 0.05$). The basilar arterial narrowing peaked on day 5 (70.1 ± 2.8% of the control, $P < 0.05$) and then persisted to day 7 (76.1 ± 2.6% of the control, $P < 0.05$) (Figure 1).

3.2. mRNA Expression of RLN1 and RXFP1 in the Rabbit Basilar Artery after SAH and Microarray Analysis of the Rabbit Basilar Artery. The three most significantly upregulated

Table 1: The top three most highly upregulated genes in the rabbit basilar artery after subarachnoid hemorrhage identified with microarray analysis.

	Day 3		Day 5		Day 7	
	Gene	$\log_2$FC	Gene	$\log_2$FC	Gene	$\log_2$FC
1	SAA	6.02	RLN1	4.73	RLN1	4.82
2	SAA3P	5.88	SAA3P	3.50	SAA3P	3.93
3	HP	5.78	HP	3.40	HP	3.47

SAA: serum amyloid A; SAA3P: serum amyloid A3 protein; HP: haptoglobin; RLN1: relaxin-1; $\log_2$FC: $\log_2$ fold change.

genes in the rabbit basilar artery after SAH on days 3, 5, and 7 compared to day 0 were identified from our previous microarray data (GEO accession number GSE44910) and are listed in Table 1 [20]. Among all investigated differentially expressed genes, *RLN1* was the most upregulated gene on days 5 and 7. The $\log_2$ fold changes for *RLN1* were 2.1, 4.7, and 4.8 on days 3, 5, and 7, respectively. We focused on *RLN1* for further investigation because the *RLN1* mRNA was markedly upregulated on days 5 and 7 when delayed cerebral vasospasm became more severe. To confirm the *RLN1* mRNA expression seen in the microarray, qRT-PCR was performed and showed that the expression of *RLN1* mRNA was significantly upregulated on day 7 compared to day 0 (Figure 2(a)). Next, we investigated the expression of *RXFP1*, which is a specific receptor for *RLN1* that is not present on the microarray chip we used. Quantitative RT-PCR revealed that *RXFP1* mRNA was significantly downregulated on days 3 and 7 (Figure 2(b)). *RLN1* mRNA was gradually upregulated after SAH, whereas *RXFP1* mRNA was persistently downregulated immediately after SAH.

3.3. Time Course of Changes in the Relaxin Concentration in CSF and Serum in Rabbits after SAH. We performed ELISA to measure the relaxin concentration in the rabbit CSF and serum after SAH. Before SAH induction, the relaxin concentration in CSF was 16.5 ± 5.6 pg/mL. After SAH, the relaxin concentration in CSF increased gradually and peaked on day 7. The relaxin concentrations in CSF on days 3, 5, and 7 were 27.9 ± 11.8 pg/mL, 58.3 ± 6.9 pg/mL, and 79.9 ± 15.9 pg/mL, respectively, with significant elevation on days 5 and 7 (Figure 3(a)). The time course of elevation of the relaxin concentration in CSF was consistent with the upregulation of RLN1 mRNA in the basilar artery.

The relaxin concentration in serum was 161.5 ± 29.1 pg/mL before induction of SAH, and on days 3, 5, and 7, the concentrations were 219.5 ± 38.7 pg/mL, 237.5 ± 40.6 pg/mL, and 233.9 ± 46.7 pg/mL, respectively. No significant difference was found between control and SAH samples at any time (Figure 3(b)).

3.4. Localization and Expression of RXFP1 in the Rabbit Basilar Artery. To assess the localization of RXFP1 expression in the rabbit basilar artery, immunohistochemical analysis of RXFP1 was performed. RXFP1-positive cells were observed in all layers of the rabbit basilar artery, especially in the media tunica of both control (day 0) and SAH (days 3, 5, and 7) samples (Figure 4(a)). Immunoreactivity for RXFP1 was seen on day 0, indicating that RXFP1 was constitutively expressed in normal basilar arteries of rabbits.

Densitometry demonstrated that RXFP1 immunoreactivity on days 3, 5, and 7 was significantly decreased compared to day 0. Arbitrary densitometric units for each day were as follows: 1,008,818.6 ± 60,874.8 on day 0, 489,367.1 ± 139,762.8 on day 3 ($P < 0.05$), 359,591.3 ± 76,946.9 on day 5 ($P < 0.05$), and 427,643.9 ± 147,666.8 on day 7 ($P < 0.05$) (Figure 4(b)). The time course of RXFP1 immunoreactivity was consistent with that of *RXFP1* mRNA expression.

3.5. Phosphorylation of MLCs during Endothelin-1 Stimulation of Cultured HBVSMC. Phos-tag SDS-PAGE followed by immunoblot detection with anti-MLC antibody yielded three bands, as previously reported [21]. The upper, middle, and lower bands were di-, mono-, and nonphosphorylated forms of MLC, respectively. HBVSMC contained 71.0 ± 3.5% mono- and di-phosphorylated forms of MLC before endothelin-1 stimulation ($n = 3$).

HBVSMC preincubated with relaxin contained 68.9 ± 3.2% phosphorylated MLC before endothelin-1 stimulation ($n = 3$). No significant difference in the resting level of MLC phosphorylation (the sum of di- and mono-phosphorylation) was found between HBVSMC preincubated with or without relaxin. Endothelin-1 (100 nmol/L) significantly increased the level of MLC phosphorylation in HBVSMC preincubated with (82.1 ± 1.6%, $P < 0.05$) and without (80.4 ± 1.4%, $P < 0.05$) relaxin 5 minutes after stimulation ($n = 3$). Ten minutes after endothelin-1 stimulation, the level of MLC phosphorylation was still significantly elevated in HBVSMC not preincubated with relaxin (79.5 ± 1.3%, $P < 0.05$), whereas it declined in HBVSMC preincubated with relaxin (78.7 ± 1.1%, $P > 0.05$) ($n = 3$). Then, the level of MLC phosphorylation declined in HBVSMC preincubated both with (72.9 ± 3.7%, $P > 0.05$) and without (75.4 ± 1.0%, $P > 0.05$) relaxin 30 minutes after endothelin-1 stimulation ($n = 3$) (Figure 5).

4. Discussion

In this study, we demonstrated that relaxin and RXFP1 are expressed in the rabbit basilar artery and that their expression was altered after SAH. *RLN1* mRNA was gradually upregulated after SAH, whereas *RXFP1* mRNA and protein were downregulated just after SAH. Moreover, the relaxin concentration in CSF was elevated after SAH, and the time course of elevation of relaxin in CSF was consistent with the progression of cerebral vasospasm. We also demonstrated that pretreatment with human recombinant relaxin-2 reduced the sustained MLC phosphorylation that was caused by endothelin-1 stimulation in HBVSMC, suggesting that relaxin inhibits the endothelin-1-induced sustained contraction in the cerebral arteries. These data suggest that upregulation of relaxin-1 and downregulation of RXFP1 play an important role in the development of cerebral vasospasm.

Relaxin affects several organs and exerts multiple effects including vasodilatation, antifibrosis, and anti-inflammation,

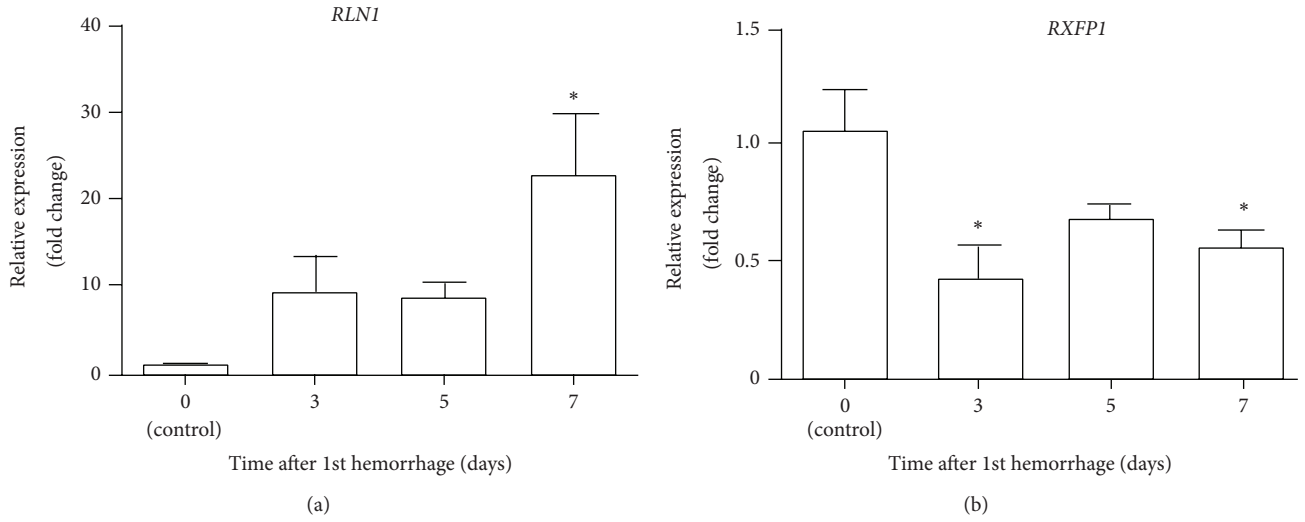

Figure 2: Time course of changes in mRNA expression of *RLN1* and *RXFP1* in the rabbit basilar artery after subarachnoid hemorrhage (SAH). Quantitative real-time polymerase chain reaction analysis of *RLN1* (a) and *RXFP1* (b) mRNA expression in the basilar artery after SAH. Data are the means ± SEM ($n = 5$). The level of mRNA expression on day 0 was considered 1.0. $^{*}P < 0.05$ versus day 0 (control).

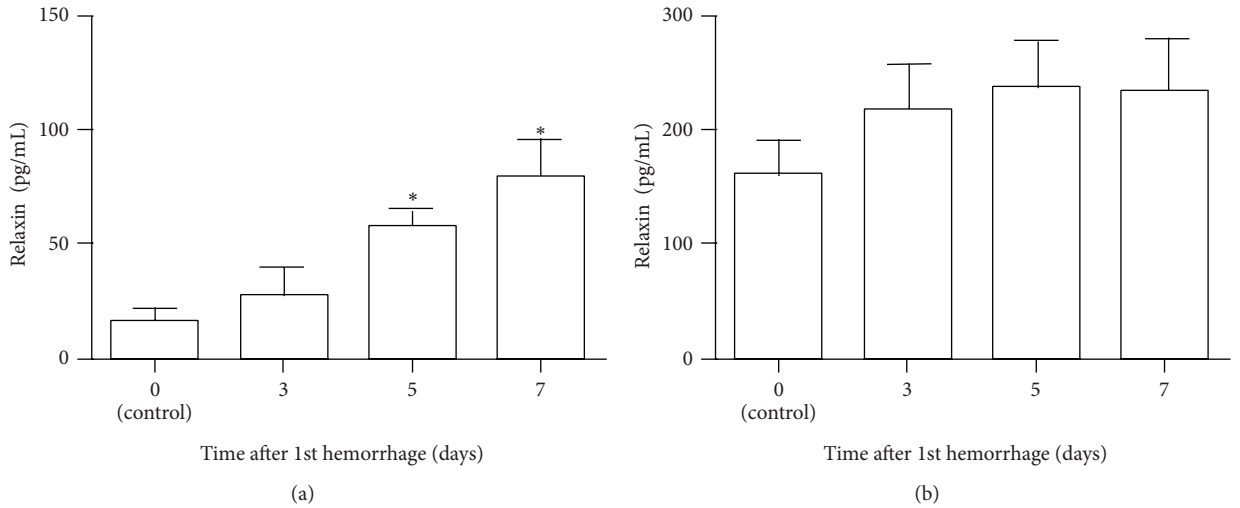

Figure 3: Time course of changes in the concentration of relaxin in cerebrospinal fluid (CSF) and serum in rabbits after subarachnoid hemorrhage (SAH). Enzyme-linked immunosorbent assay analysis of the relaxin concentration in the CSF (a) and serum (b) of rabbits after SAH. Data are the means ± SEM (CSF $n = 6$, serum $n = 15$). $^{*}P < 0.05$ versus day 0 (control).

and this peptide maintains organ blood flow via RXFP1 [8, 14, 22]. Several studies have demonstrated that relaxin dilates arteries [23, 24]. Our in vitro data demonstrated that relaxin reduced the sustained MLC phosphorylation after endothelin-1 stimulation. Therefore, relaxin has the potential to dilate cerebral arteries. Immunohistochemistry and qRT-PCR demonstrated that downregulation of *RXFP1* occurred 3 days after SAH and persisted to day 7 in the rabbit basilar artery. A previous study in rats reported that downregulation of RXFP1 expression in the uterus reduces the smooth muscle relaxation activity of relaxin [25]. Therefore, the vasodilatory effect of relaxin on cerebral arteries may be reduced in the rabbit basilar artery after SAH. A functional decrease in relaxin activity caused by RXFP1 downregulation seems to be involved in the development of cerebral vasospasm. Because relaxin exerts multiple effects on several organs, further studies are needed to elucidate the effects of relaxin on cerebral arteries.

The mechanism of regulation of RXFP1 expression in the artery remains unknown. Expression of *RLN3* mRNA may contribute to the mechanism of *RXFP1* downregulation in rat heart [6]. In this study, *RLN3* mRNA was not detected in the rabbit basilar artery at any time point investigated (data not shown). Moore et al. proposed that β-adrenergic receptor activation, especially β1-adrenergic receptors, suppresses the expression of *RXFP1* mRNA in rat heart [26]. Adrenaline is a representative spasmogen [1] and is increased in plasma and CSF after SAH [27]. The elevated expression of adrenaline

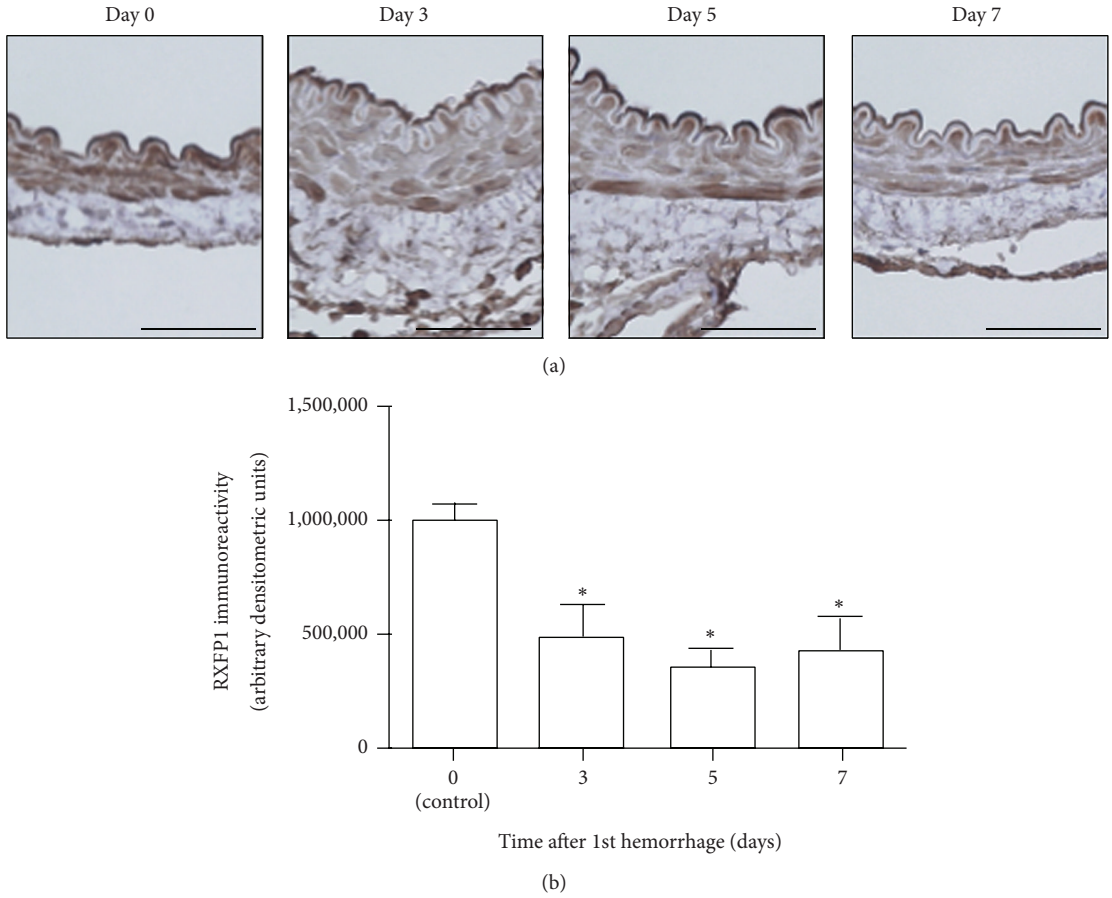

Figure 4: Time course of changes in RXFP1 expression in the rabbit basilar artery after subarachnoid hemorrhage (SAH). (a) Representative images of immunohistochemical staining for RXFP1 on cross sections of rabbit basilar arteries after SAH (scale bars, 50 μm). (b) A summary of densitometric analysis of immunohistochemical staining for RXFP1. Data are the means ± SEM ($n = 3$ per group). $^{*}P < 0.05$ versus day 0 (control).

may participate in the mechanism of RXFP1 downregulation in the basilar arteries after SAH.

Luteral relaxin expression is regulated by luteinizing hormone, chorionic gonadotrophin, and basic fibroblast growth factor [28]. However, the mechanism of regulation of the expression of arterial-derived relaxin remains elusive. Our data demonstrated that downregulation of RXFP1 was followed by upregulation of relaxin-1. One explanation for this finding may be that a receptor-ligand feedback mechanism exists to regulate the expression of the ligand. However, further research is needed to identify the mechanism of regulation of expression of relaxin-1 and RXFP1 in the cerebral arteries after SAH.

The concentration of relaxin in CSF was significantly elevated after SAH, and the time course was consistent with that of *RLN1* mRNA expression in the rabbit basilar artery. In contrast, the relaxin concentration in serum was not elevated after SAH. The relaxin concentration in serum is affected by systemic conditions such as pregnancy, heart failure, and cancer [3, 29, 30]. The relaxin concentration in CSF may be affected by intracranial conditions after SAH. Arterial-derived relaxin is produced locally and acts in an autocrine/paracrine manner [8, 13]. Therefore, the elevated relaxin concentration in CSF indicates that relaxin is produced in the basilar arterial wall and secreted into the CSF. Furthermore, the time course of the relaxin concentration in CSF was correlated with the progression of cerebral vasospasm after SAH. Thus, the relaxin concentration in CSF may be a potential biomarker for detecting the presence of cerebral vasospasm after SAH.

In conclusion, we demonstrated that *RLN1* mRNA was gradually upregulated after SAH, whereas *RXFP1* mRNA and protein were downregulated just after SAH. The relaxin concentration in CSF was significantly elevated after SAH, and the time course was consistent with that of expression of *RLN1* mRNA in the rabbit basilar artery. These findings suggest that expression and signaling of relaxin and RXFP1 participate in the development of cerebral vasospasm after SAH. Moreover, downregulation of RXFP1 may cause a functional reduction in relaxin activity in cerebral arteries during vasospasm. Further studies are needed to clarify the role of relaxin in the development of cerebral vasospasm.

Conflict of Interests

The authors declare that they have no conflict of interests.

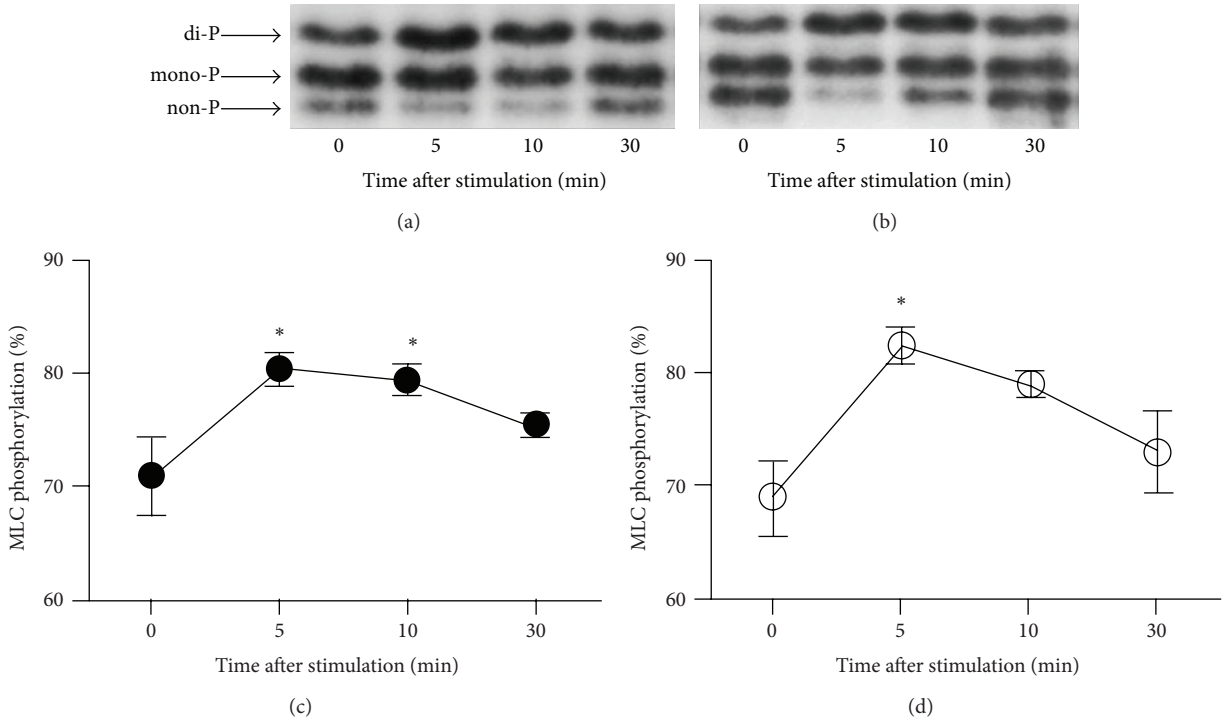

Figure 5: Time course of changes in myosin light chain (MLC) phosphorylation induced by endothelin-1 in human brain vascular smooth muscle cells (HBVSMC). Representative immunoblot analysis of MLC phosphorylation in HBVSMC preincubated without (a) and with (b) human recombinant relaxin-2 (H2 relaxin). The upper, middle, and lower bands detected with anti-MLC antibody represented di-, mono-, and nonphosphorylated forms of MLC, respectively. Summary of the level of MLC phosphorylation (percent of total MLC) induced by 100 nmol/L endothelin-1 at four time points: just before relaxin stimulation (0 minutes) and 5, 10, and 30 minutes after relaxin stimulation ((c) preincubated without H2 relaxin, (d) preincubated with H2 relaxin). The data are the means ± SEM ($n = 3$). $^*P < 0.05$ versus 0 minutes.

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Research Article

Patient Outcomes following Subarachnoid Hemorrhage between the Medical Center and Regional Hospital: Whether All Patients Should Be Transferred to Medical Centers

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Subarachnoid hemorrhage (SAH) is a critical illness that may result in patient mortality or morbidity. In this study, we investigated the outcomes of patients treated in medical center and nonmedical center hospitals and the relationship between such outcomes and hospital and surgeon volume. Patient data were abstracted from the National Health Insurance Research Database of Taiwan in the Longitudinal Health Insurance Database 2000, which contains all claims data of 1 million beneficiaries randomly selected in 2000. The International Classification of Diseases, Ninth Revision, subarachnoid hemorrhage (430) was used for the inclusion criteria. We identified 355 patients between 11 and 87 years of age who had subarachnoid hemorrhage. Among them, 32.4% (115/355) were men. The median Charlson comorbidity index (CCI) score was 1.3 (SD ± 0.6). Unadjusted logistic regression analysis demonstrated that low mortality was associated with high hospital volume (OR = 3.21; 95% CI: 1.18–8.77). In this study, we found no statistical significances of mortality, LOS, and total charges between medical centers and nonmedical center hospitals. Patient mortality was associated with hospital volume. Nonmedical center hospitals could achieve resource use and outcomes similar to those of medical centers with sufficient volume.

1. Introduction

The annual subarachnoid hemorrhage incidence is 7–20/100 000/y [1–5]. Mortality rates of this devastating disease range from 32% to 50% [3, 6–8]. The overall prognosis of patients with subarachnoid hemorrhage remains poor with nearly half of the survivors diagnosed with sequelae [8, 9], which is associated with substantial financial burdens on the healthcare system. Acute subarachnoid hemorrhage patients are generally sent to the nearest hospitals, although some of them may request a transfer to medical centers. Because numerous regions lack medical centers, patients typically remain at nonmedical center hospitals to receive treatment.

Most medical centers in Taiwan are training hospitals, and young trainees and residents remain in a hospital for several years, until their abilities and experience are sufficient for transferring to other hospitals. Therefore, compared with nonmedical center hospitals, whether the outcomes and medical expenses in medical centers are more favorable is uncertain. Standardized process-of-care measures might play a role in optimizing quality and efficiency, regardless of hospital or surgeon volume [10].

Acute subarachnoid hemorrhage is a disease that can be fatal if it occurs abruptly. Clinical decision-making and policy-making for subarachnoid hemorrhage are challenging and require effective planning and medical care. In this study, we used nationwide population-based data on all hospitalizations for subarachnoid hemorrhage between 2000 and 2009, from the Taiwan National Health Insurance Research Database (NHIRD), to analyze the associations between outcome and hospital level. The underlying assumption is that medical centers may achieve enhanced outcome and low cost in treating subarachnoid hemorrhage. The second aim of this population-based study was to explore the predictors of hospital resource use and mortality rates in a population of patients who had acute subarachnoid hemorrhage.

2. Materials and Methods

2.1. Data Source. The National Health Insurance (NHI) program, established in March 1995, is the only public insurance system for the entire population of Taiwan and is a universal healthcare system covering 99% of the country's population of 23 million. Patient data were abstracted from a subdataset of the NHIRD in the Longitudinal Health Insurance Database 2000, which contains all claims data (from 1996 to 2009) of 1 million beneficiaries randomly selected in 2000. Between the sample groups and all enrollees, no significant difference existed in age, gender, or health care costs. The encrypted secondary database contains patient-level demographic and administrative information including sex, birthdates, dates of admission and discharge, hospital level of the institutions providing services, the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) diagnosis (up to 5) and procedure (up to 5) codes, status of patient discharge (recovered, died, or transferred out), and hospital charges of all medical expenses. This program provides a highly reliable database for researchers. The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University.

2.2. Study Sample. All patients included in the study had been discharged from a hospital included in the NHIRD during the 10-year period between 2001 and 2009. International Classification of Diseases, Ninth Revision, Clinical Modification classification code subarachnoid hemorrhage (430) was used for the inclusion criteria. The exclusion criteria included ICD-9-CM 800.0–801.9, 803.0–804.9, 850.0–854.1, and 873.0–873.9 (head injury).

2.3. Variables. In-hospital mortality, total charges during hospitalization, and hospital LOS were used as the outcome variables of this study. Patient age, sex, and the CCI score were used as covariates. Hospital-level covariates used in adjustment included geographical region (Northern, Central, and Southern Taipei and Eastern Kao-Ping) and accreditation level (academic medical center, regional, and district). We further categorized an "academic medical center" as a medical hospital and a "regional or district hospital" as a nonmedical center hospital.

The mortality rate was defined as being within 30 days of hospital admission, as suggested by the Centers for Medicare and Medicaid Services. Because the NHI in Taiwan is a single-payer health program, the only reason for being withdrawn from NHI coverage within 30 days of hospital admission would be that the patient had expired (the other 2 conditions for withdrawal from the NHI coverage, being incarcerated for over 2 month or disappearing for over 6 month, would not be possible reasons for withdrawal within 30 days of hospital admission) [11].

Because the hospital and surgeon volumes in our data did not constitute a normal distribution and the volume categories developed by Peterson et al. [12] may not optimally characterize the distribution of our study, we simply divided the distribution of the volumes during the study period into approximately 2, according to hospital volume (less than 30 and more than 30) and surgeon volume (less than 2 and more than 2). The lowest volume of surgeons and hospitals served as the reference group.

2.4. Statistical Analysis. The patient characteristics were analyzed between the medical center hospital and the nonmedical center hospital to determine differences in age, sex, CCI, and surgical clipping rate. After confirming that no differences in patient characteristics existed between the hospital levels, we further analyzed the outcomes regarding mortality, medical expenditure, and hospital LOS. The mortality of these patients was compared for differences in their demographic characteristics and hospital level by using bivariate analysis. Categorical variables were compared using the χ^2 or a Mann-Whitney 2-independent-sample test. Unadjusted and adjusted logistic regression analyses were used to estimate the odds ratio (OR) and its 95% confidence interval (CI) between mortality and age, sex, CCI, hospital levels, and hospital and surgeon volume. A value of $P < .05$ was considered statistically significant. All statistical calculations were performed using the Statistical Package for Social Sciences for Windows (SPSS for Windows 19.0).

3. Results

3.1. Patient Characteristics. We identified 355 patients between 11 and 87 years of age who had acute subarachnoid hemorrhage. Among them, 32.4% (115/355) were men. The median CCI score was 1.3 ± 0.6. Of the patients included in the study, 25 died while being in the hospital (7.0%), LOS was 23.1 ± 13.2 days, and the total admission charge was NT$365848 ± NT$221534 (US$1.00 = NT$30.27 in 2011) (Table 1).

3.2. Patient Characteristics between Hospital Levels. In the univariable analysis, fewer patients were treated in nonmedical centers than in medical centers (101 versus 254). The 2-independent-sample test analysis and the Pearson χ^2 test showed no significant differences between medical and nonmedical center hospitals in patient characteristics (sex, age, surgical clipping rate, and CCI). Outcomes including in-hospital mortality, LOS, and total admission charges between

Table 1: Patient demographics of study population and overall hospital characteristics for brain aneurysm surgery[a].

Characteristic	Finding
Patients, total number	355
Male sex	115/355 (32.4%)
Age, median (SD), y	57.2 (14.8)
CCI, median (SD)	1.3 (0.6)
Hospital characteristics	
30-day mortality	25/355 (7.0%)
LOS, median (SD), d	23.1 (13.2)
Total charges, median (SD), $NTD	365,848 (221,534)

LOS: length of stay; CCI: Charlson index comorbidity score.
[a]Unless indicated otherwise, data are reported as number of patients in the relevant category/number of patients possible in the category (percentage). US$1.00 = NT$30.27 in 2011.

the hospital levels had no statistical differences (Table 2). One hundred percent of the nonmedical hospitals had a volume exceeding 30 and 40.2% of medical centers had a volume exceeding 30. Surgeon volume of nonmedical centers was 36.6% more than 2 and 46.5% of medical centers had a surgeon volume exceeding 2 during the study period.

3.3. Outcomes between Hospital Levels. The unadjusted and adjusted logistic regression analyses (enter model) were used to estimate in-hospital mortality. Unadjusted logistic regression analysis demonstrated that low mortality was associated with hospital volume (OR = 3.21; 95% CI: 1.18–8.77). After we adjusted for patient's sex, surgeon volume, hospital level (medical center versus nonmedical center hospital), and CCI, significant associations existed in hospital volume ($P = .024$; OR = 0.277; 95% CI: 0.091–0.842) with the 30-day mortality (Table 3).

We observed a significant association between total charges and LOS ($P < .001$) after adjusting for patient age, sex, mortality, and hospital level using linear regression analyses. Medical centers were associated with a trend toward increased total charges, although this was not statistically significant after adjustment. After we adjusted for patient age, sex, mortality, and hospital level by using linear regression analyses, LOS was not associated with any variants.

4. Discussion

We used nationwide population-based data to evaluate the difference between mortality and LOS, as well as the total charges of acute subarachnoid hemorrhage treated between medical centers and nonmedical center hospitals (regional and district). We observed no statistical significance in mortality, LOS, or total charges between medical centers and nonmedical center hospitals. However, patient mortality was associated with hospital volume.

Acute subarachnoid hemorrhage is a complex disease that is associated with high risk of mortality and morbidity. Medical centers have more medical resources and facilities than other levels of hospitals, including sophisticated intensive care units, live support equipment, and specialist personnel. Therefore, centralized patients might have beneficial outcomes. However, in this study, we found that patient mortality was associated with hospital volume rather than hospital level. The outcome of acute subarachnoid hemorrhage treated in nonmedical center hospitals yielded results similar to those of medical centers. In Taiwan, only board-certified neurosurgeons can perform acute subarachnoid hemorrhage surgery and neurosurgeons should be trained in medical centers for more than 7 years before obtaining board certification after full training for a national examination. Thus, neurosurgeons practicing in regional or district hospitals have full capability and clinical experience for patient care and surgical skills.

The mortality rate for patients with acute subarachnoid hemorrhage was 7% in this study, similar to the 8.8%–48.5% in the United States, and 9.6% in the Japan study that investigated the relationships between case volume and outcome [13–18]. Numerous studies have shown the relationships between hospital volume and outcome in cerebral aneurysm clipping [19, 20]. Leake et al. evaluated the US National Inpatient Sample for the period 2001–2008 for outcomes and trends in patient admissions for treating subarachnoid hemorrhage at high- and low-volume centers [19], concluding that the treatment of ruptured cerebral aneurysms increasingly occurs at high-volume centers in the United States. They observed enhanced outcomes associated with treating these lesions at high-volume centers [19]. However, another study conducted in Japan did not favor the same conclusion [20]. Hattori et al. analyzed a nationwide study to investigate the relationships between case volume and outcome in cerebral aneurysm clipping surgery performed in 2003. A total of 11974 clipping procedures were included in the report. The final data showed that a greater case volume did not correlate with a favorable outcome, which indicated that higher volume in medical centers than in nonmedical center hospitals may not influence the outcomes [20]. In our study, we found that patient mortality was associated with hospital volumes rather than hospital level. Cross et al. also found that high-volume subarachnoid hemorrhage treatment centers might improve overall survival [21]. We suggest that volume is a crucial factor because of medical centers having more patients; however, a standardized quality of care in nonmedical center hospitals could improve outcomes if their neurosurgeons undergo effective training.

In this study, we found no significant reduction of LOS, mortality rate, and total charges between the 2 hospital levels. One of the reasons may be that patients with more complications were transferred to medical centers. However, the Taiwan health care system allows patients free access to any hospital of their choice; thus, medical centers attract difficult cases and have numerous less severe patients. In addition, subarachnoid hemorrhage patients might not be regionalized before transferring to a hospital; thus, patients are generally sent to the nearest hospital. This practice leaves little room for deliberate patterns of selective referral to either specific hospitals or attending physicians. Moreover, patients sent to nonmedical center hospitals might not be able to be transferred because of critical conditions. We observed no differences in medical expenditure between the hospital levels. To manage a higher medical workload, medical

Table 2: Univariable analysis of patient demographics and hospital characteristics for brain surgery of aneurysm stratified by hospital status[a].

Characteristic	Hospitals of other levels ($n = 101$)	Medical center ($n = 254$)	P
Female (%)	68/101 (67.3)	172/254 (67.7)	0.944[b]
Age, median (SD), y	58.4 (13.8)	56.8 (15.2)	0.355[c]
CCI, median (SD)	1.3 ± 0.5	1.3 ± 0.7	0.814[c]
Surgical clipping (%)	58/101/(57.4)	143/254 (56.3)	0.847[b]
Hospital characteristics			
In-hospital mortality (%)	8/101 (7.9)	17/254 (6.7)	0.683[b]
LOS, median (SD), d	23.2 (12.5)	23.2 (13.5)	0.991[c]
Total charges, median (SD), $NTD	356,637 ± 174,594	369,511 ± 237,851	0.622[c]

LOS: length of stay; CCI: Charlson index comorbidity score.
[a]Unless indicated otherwise, data are reported as number of patients in the relevant category/number of patients possible in the category (percentage). [b]Pearson χ^2 test.
[c]Two-independent-sample test (Mann-Whitney U). US$1.00 = NT$30.27 in 2011.

Table 3: Independent risk factors associated with mortality.

Factor	Odds ratio	P value	95% confidence interval
Hospital volume	0.277	0.024	0.091–0.842
Surgeon volume	1.700	0.277	0.653–4.423
Hospital levels	0.648	0.374	0.249–1.686
Gender	0.921	0.864	0.362–2.348
Age	1.028	0.094	0.995–1.061
CCI	1.453	0.135	0.890–2.374

The risk factors included in the logistic regression model were surgeon volume, hospital volume, hospital levels, gender, age, and CCI.

centers typically employ more physicians and nurses than nonmedical center hospitals do. The staff-to-patient ratio may often be higher in larger hospitals. Subspecialists, including cerebrovascular neurosurgeons, interventional neuroradiologists, neuroanesthesiologists, and neurointensivists, are also more likely to be included in the staff. Patients admitted to medical centers may have more routine checkups and exams following visits with specialists. Therefore, although medical centers might have more treatment experience and might reduce certain aspects of medical expenditure during management, they spend more on expansion, which eventually makes no difference with nonmedical center hospitals.

Surgical skills improve with increased experience, but they are also affected by surgeon training. In Taiwan, the Taiwan Neurosurgical Society conducts neurosurgical specialist training, which has established high standards for neurological surgeries, including cerebral aneurysm clipping. The neurosurgeon should be trained in medical centers for more than 7 years and become board certified after a national examination. In Taiwan, neurosurgeons practicing at nonmedical center hospitals should have more confidence in treating patients without transfer. Therefore, by providing an effective training system, the performance of nonmedical center hospitals in treating subarachnoid hemorrhage might not be inferior to that of medical centers if neurosurgeons demonstrate sufficient confidence.

Limitations. Despite the strengths of our study using a national database, the findings must be interpreted with caution because of the following limitations. The NHIRD contains a complete representation of all cases admitted to all hospitals of Taiwan. However, this study is a retrospective review of the NHIRD data; therefore, there is significant potential for selection bias and uncontrollable factors that could influence the outcomes of medical center and nonmedical center hospitals. We attempted to address this by using logistical analysis to adjust for several patient-specific and hospital-specific factors, and the purpose of this study was not to look for those factors. A further limitation of our study is the lack of data in the NHIRD on aneurysm-specific characteristics, such as aneurysm location and size, and clinical data, including the severity of SAH and patient's Glasgow Coma Scale score. However, in our study, the CCI exhibited no difference between the hospital levels, which may present no statistical difference in patient comorbidity between hospital levels. Certain information, such as times from bleeding to surgery, rebleeding, and the use of lumbar drainage, was not recorded in the database. Therefore, we cannot provide these outcomes in this study. Another limitation of this study is that we relied exclusively on claims data, which may result in potential disease classification bias. However, we are confident in assuming that the patient characteristics were similar at the hospital levels, which may decrease the bias in our results.

5. Conclusion

We observed no statistically significant measures of mortality, LOS, or total charges between medical centers and nonmedical center hospitals of patients with acute subarachnoid hemorrhage. Patient mortality was associated with hospital volume. Nonmedical center hospitals with sufficient hospital volumes could achieve similar resource use and outcomes as medical centers in treating subarachnoid hemorrhage.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

The first two authors made equal contributions to this work and are equally considered to be first authors.

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Research Article

The Role of Microclot Formation in an Acute Subarachnoid Hemorrhage Model in the Rabbit

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Background. Microvascular dysfunction and microthrombi formation are believed to contribute to development of early brain injury (EBI) after aneurysmal subarachnoid hemorrhage (SAH). *Objective.* This study aimed to determine (i) extent of microthrombus formation and neuronal apoptosis in the brain parenchyma using a blood shunt SAH model in rabbits; (ii) correlation of structural changes in microvessels with EBI characteristics. *Methods.* Acute SAH was induced using a rabbit shunt cisterna magna model. Extent of microthrombosis was detected 24 h post-SAH ($n = 8$) by fibrinogen immunostaining, compared to controls ($n = 4$). We assessed apoptosis by terminal deoxynucleotidyl transferase nick end labeling (TUNEL) in cortex and hippocampus. *Results.* Our results showed significantly more TUNEL-positive cells (SAH: 115 ± 13; controls: 58 ± 10; $P = 0.016$) and fibrinogen-positive microthromboemboli (SAH: 9 ± 2; controls: 2 ± 1; $P = 0.03$) in the hippocampus after aneurysmal SAH. *Conclusions.* We found clear evidence of early microclot formation in a rabbit model of acute SAH. The extent of microthrombosis did not correlate with early apoptosis or CPP depletion after SAH; however, the total number of TUNEL positive cells in the cortex and the hippocampus significantly correlated with mean CPP reduction during the phase of maximum depletion after SAH induction. Both microthrombosis and neuronal apoptosis may contribute to EBI and subsequent DCI.

1. Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disease with high mortality and disability rates [1]. Intensive research in recent years revealed many different causes of SAH, including cerebral vasospasm, early brain injury (EBI) mediated by impaired microcirculatory function, microthrombosis, cortical spreading depression, oxidative stress, inflammation, and apoptosis. All of these conditions cause delayed cerebral ischemia (DCI) and thereby influence clinical outcome [1–6]. However, the relationship of microthrombi formation to early brain injury and neuronal apoptosis after SAH still remains unclear. To model the physiological situation in humans with aneurysmal SAH [7], this study investigated the association between early injury after SAH, microclot formation, and apoptosis in an extra-ntracranial blood shunt model in the rabbit.

2. Materials and Methods

2.1. Study Design. The study was incorporated as a subproject of ongoing experimental studies and performed in accordance with the National Institutes of Health guidelines

for the care and use of experimental animals and with the approval of the Animal Care Committee of the Canton of Bern, Switzerland (approval no. 107/09). Of 12 three-month-old female New Zealand rabbits weighing 3.3–4.6 kg, four animals served as sham-operated controls. In eight animals, experimental SAH was performed as described below. The animals were housed in groups (two to four animals per cage) at 22–24°C under a 12-hour light-dark cycle with unrestricted access to food and tap water. All surgical procedures were performed under sterile conditions at the Experimental Surgical Institute, Department of Clinical Research, Bern University Hospital, Bern, Switzerland. A veterinary anesthesiologist monitored the animals during surgery and throughout anesthetic recovery.

2.2. Anesthesia, Clinical Observation, and Sacrifice. Induction of general anesthesia was performed by subcutaneous administration of ketamine (30 mg/kg; Ketalar, 50 mg/mL, Pfizer, Zurich, Switzerland) and Xylazine (6 mg/kg; Xylapan 20 mg/mL, Vetoquinol, Bern, Switzerland) and continued intravenously. Humidified oxygen was provided to the spontaneously breathing animals. The animals underwent clinical observation during anesthetic recovery (first three hours) and from then on every six hours. Euthanasia was performed 24 hours post-SAH induction under the same anesthesia as previously described, by intra-arterial bolus injection of sodium thiopental (40 mg/kg) (Pentothal, Ospedalia AG, Hünenberg, Switzerland).

2.3. SAH Induction, Instruments, and Data Acquisition. The intracerebral pressure- (ICP-) controlled blood shunt model was used to induce SAH as described previously [7, 8]. Briefly, the cisterna magna was punctured with a pediatric spinal access needle (22 G × 40 mm) and connected via pressure tube and interposed three-way stopcock to the subclavian artery. The three-way stopcock was used for blood pressure measurement and to control bleeding. Neuromonitoring including an ICP monitor catheter tip (OLM Intracranial Pressure Monitoring Kit, Camino, Model 110-4B, Camino Laboratories, San Diego, CA, USA) and two laser-Doppler flowmetry fine needle probes (MNP110XP, 0.48 mm diameter, Oxford Optronix Ltd., Oxford, UK) were positioned in the olfactory bulb and bilateral frontal lobe according to outer skull landmarks [9]. Standard cardiovascular monitoring (mean arterial blood pressure (MABP), heart rate, electrocardiogram, end-tidal CO_2, and SaO_2) was performed at a sampling rate of 100 Hz (Datex S5 Monitor GE Medical Systems Switzerland, Glattbrugg, Switzerland), transferred via the analog output interface to an analog-digital converter/data logger, stored (Biopac MP100 and acknowledge version 3.8.1; BIOPAC Systems, Inc., Goleta, CA, USA), and processed for preanalysis using scripting software (Mathworks Inc, Natick, MA, USA). Pressures were zeroed at heart level before and after each session, and pressure calibration of the AD converter and data-logging system was done once before the series started.

SAH was initiated by opening the blood shunt to let blood stream into the atlantooccipital cistern under arterial pressure. After opening the shunt, ICP increased until it reached a plateau. If this plateau phase was maintained for more than 10 seconds, the shunt was closed. The shunt was also closed if ICP decrease occurred spontaneously (no later than 30 seconds from start of the plateau phase—we therefore did not allow for potential rebleeding). Control animals underwent frontal osteotomy with ICP and cerebral blood flow (CBF) monitoring placement, as well as puncture of the cisterna magna without blood shunting. MABP, ICP, and bilateral regional cerebral blood flow rCBF were recorded for 5 minutes before (baseline) and 20 minutes after initiation of SAH (steady state).

2.4. Tissue Processing, Histology, and Immunohistochemistry. Intracardiac perfusion-fixation was carried out 24 hours after SAH-induction at room temperature with 400 mL of 0.1 M phosphate-buffered solution (PBS) followed by 400 mL fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.3). Brains were removed from the skull and cut into four blocks between the forebrain (olfactory bulb) and cerebellum, embedded in paraffin, and cut into consecutive 7 μm sections. The cut surface of block one was placed through the cortical punch defect of the ICP and rCBF probes. The first section of blocks two to four was stained with hematoxylin and eosin, and the most representative fields containing the hippocampus and basal cortex were selected for additional cuts of ten consecutive sections used for immunohistochemical analysis in order to analyze the same subsection to eliminate bias. Apoptosis was detected using terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL, Roche Diagnostics AG, Rotkreuz, Switzerland) as described above [7]. Quantitative analysis of apoptosis was performed within predefined regions of interest (ROI) of 300 μm × 300 μm on coronal sections for each hemisphere (Figure 1(b)). Thereby, 9 ROIs were used for analysis of apoptosis in the basal cortex (9 × 300 μm × 300 μm) and 3 ROIs along the hippocampal sectors CA1 and CA3 with (3 × 300 μm × 300 μm). Nuclei were counterstained with DAPI (Roche Diagnostics AG, Basel, Switzerland). Slides were visualized under a fluorescent microscope operating with a digital camera (Olympus BX 51, Olympus, Hamburg, Germany) using 2, 10, and 20x magnifications. Thereby, TUNEL red, FJB green, and DAPI blue were excited at 570–620 nm (maximum 580 nm), 450–490 nm (maximum 480 nm), and 340–380 nm (maximum 350 nm), respectively. The extent of microthrombosis was detected by fibrinogen immunohistochemistry using the Leica Bond III IHC staining system and analyzed in a blinded manner according to the schematic drawing depicted in Figure 1. For the fibrinogen immunohistochemistry, heat-induced epitope retrieval was carried out at 95°C for 20 minutes, followed by incubation with the primary antibody (polyclonal fibrinogen sheep anti-rabbit antibody; Acris, AP08879PU-N, 1:2′000, Herford, Germany) and secondary antibody (biotin-SP-conjugated AffiniPure donkey anti-sheep antibody; Jackson ImmunoResearch Laboratories, 713-065-003, 1:1′000, West Grove; USA), followed by incubation with a streptavidin-conjugated horseradish peroxidase reagent (Streptavidin-HRP, Leica Biosystems, RE7104).

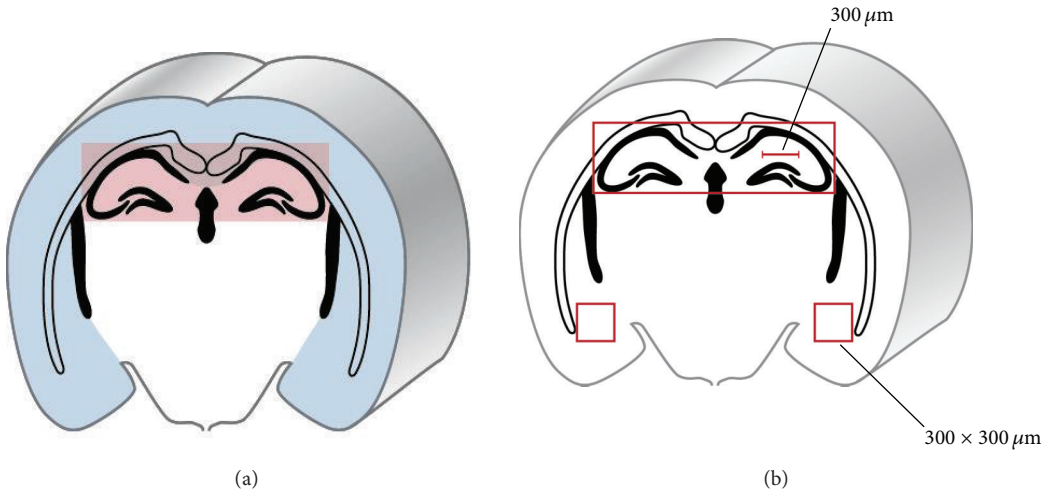

Figure 1: Microclot formation analyzed in the hippocampus and cortex. (a) Schematic drawing of regions used for analysis of fibrinogen immunostained microclots in the hippocampus (red bar) and the cortex (blue bar). (b) Specific regions of interest (ROI) from the hippocampus and cortex used for dimensional analysis.

2.5. Statistical Analysis. Data were analyzed and visualized using IBM SPSS statistical software Version 21.0 (IBM Corp., New York, NY, USA) and processed for preanalysis using Matlab scripting software (Mathworks Inc., Natick, MA, USA). Values were expressed as mean ± SEM. The differences between the normally distributed data of two groups were analyzed by Student's t-test and among three or more groups by one-way ANOVA, respectively, with Bonferroni post hoc testing. ANOVA regression analysis was used for calculation of correlations between effects of SAH on the CPP and the number of fibrinogen positive microvessels and the TUNEL-positive cells. The strength of linear correlations was expressed by the linear regression coefficient (reg coeff r) and its squared value r^2. A significance level of $P < 0.05$ was applied to all tests.

3. Results

3.1. Gross Examination of Brain and Pathophysiology. There was no mortality in this study. In general, the mortality rate is about 20%–30% due to respiratory arrest or severe bradycardia at the time of acute SAH [8]. There were no signs of cerebrospinal fluid leakage along the frontal osteotomy sites or at the site of nuchal cisterna magna puncture (data not shown). Twenty-four hours after SAH, rabbits ($n = 8$) demonstrated extensive coagulated diffuse subarachnoid blood in the chiasmatic, basal, prepontine cisterns, and cistern magna. No subarachnoid blood was observed in control animals ($n = 4$). All SAH animals demonstrated marked increases in ICP (6.2 ± 1.7 mmHg baseline versus 49.6 ± 11.9 mmHg peak; $P < 0.001$) with a corresponding decrease in bilateral rCBF (mean of both hemispheres: 36.3 ± 20.3% from baseline, $P < 0.001$) and the CPP (32.3 ± 15.0% of baseline, $P < 0.001$) within the first three minutes after induction of SAH. The ICP returned within 20 minutes to a steady state that was slightly higher than baseline but was not statistically different from baseline values (baseline: 6.2 ± 1.7 mmHg, steady state: 19.4 ± 4.3 mmHg; $P = 0.051$). Accordingly, both rCBF and CPP recovered to a state that was not significantly below baseline levels (mean of both hemispheres, rCBF: 76.8 ± 15.2% of baseline, $P = 0.18$; CPP: 81.2 ± 9.6% of baseline, $P = 0.15$). The mean arterial blood pressure remained unchanged throughout. A summary of pathophysiological characteristics is provided in Table 1.

3.2. Immunohistochemistry and Analyses. Fibrinogen staining showed distinct microclot formation in vessels of the hippocampus (Figures 2(a) and 2(c)) and cerebral cortex (Figures 2(b) and 2(d)) after SAH ((a), (b)) compared to control animals ((c), (d)). Immunohistochemistry analysis revealed a significant increase of the number of TUNEL-positive cells in both cerebral cortex and hippocampus (Figure 3(a)). Namely, there were 68 ± 8 TUNEL-positive cells in the cortex after SAH compared to 36 ± 2 cells in the control animals (differences between means 32 ± 11; $P = 0.017$). In the hippocampus, there were 115 ± 13 TUNEL-positive cells after SAH compared to 58 ± 10 positive cells in the control animals (differences between means 58 ± 20; $P = 0.016$). Taking into account the differences in the density of neurons in the hippocampus and the cerebral cortex, immunohistochemistry analyses showed in the hippocampus 115 ± 13 TUNEL-positive cells after SAH compared to 68 ± 8 positive cells in the cerebral cortex (differences between means 48 ± 15; $P = 0.014$). In control animals, there were 58 ± 10 TUNEL-positive cells in the hippocampus compared to 36 ± 2 in the cerebral cortex (differences between means 22 ± 9; $P = 0.13$).

A tendency towards increased mean number of fibrinogen-positive microvessels in cerebral cortex was noted (9 ± 2 fibrinogen positive cells after SAH compared to 2 ± 1 positive cells in control animals; $P = 0.06$). There was a significant increase in fibrinogen-positive microvessels in the hippocampus (9 ± 2 for SAH compared to 2 ± 1 in sham controls, $P = 0.03$) (Figure 3(b)).

Table 1: Pathophysiological characteristics of SAH animals ($n = 8$)[a].

Time point	MABP (mmHg)	ICP (mmHg)	Relative CPP (% of BL)	rCBF of both hemispheres (% of BL)
Baseline	68.4 ± 6.1	6.2 ± 1.7	100	100
Peak	69.5 ± 7.8	49.6 ± 11.9*†	32.3 ± 15.0*†	36.3 ± 20.3*†
Steady state	70.3 ± 5.3	19.4 ± 4.3	81.2 ± 9.6	76.8 ± 15.2

Abbreviations: BL = baseline; CPP = cerebral perfusion pressure; ICP = intracranial pressure; MABP = mean arterial blood pressure; rCBF = regional cerebral blood flow.
[a]Values are expressed as mean ± SD.
*Significantly different compared to baseline values ($P < 0.001$).
†Significantly different compared to steady-state values ($P < 0.001$).

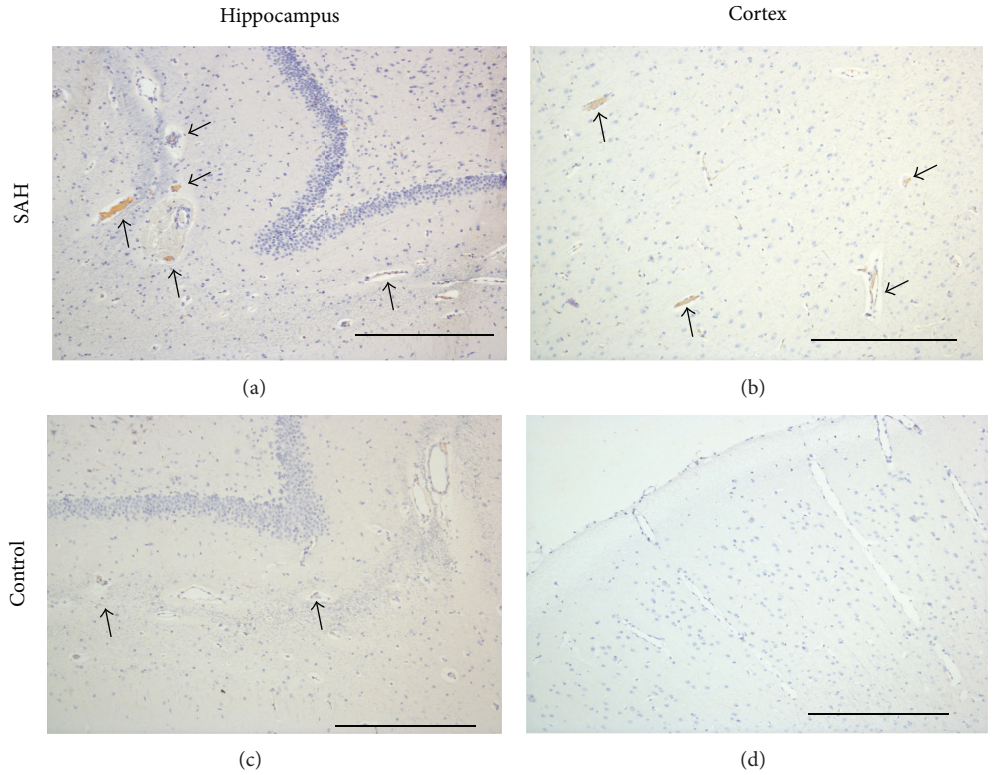

Figure 2: Microclot formation after induction of subarachnoid hemorrhage. Representative images showing fibrinogen positive vessels (brown staining; black arrows) in the hippocampus ((a), (c)) and cortex ((b), (d)) after SAH ((a), (b)) and in controls ((c), (d)). Scale bars = 400 μm.

3.3. Correlation between Apoptosis and Microclot Formation. There was no correlation between microclot formation (number of fibrinogen positive clots) and apoptosis (number of TUNEL positive cells) in the cerebral cortex (reg coeff $r = 0.31$, $r^2 = 0.094$, $P = 0.3$; Figure 3(c)) or the hippocampal region (reg coeff $r = 0.45$, $r^2 = 0.2$, $P = 0.14$; Figure 3(d)).

3.4. Correlation between CPP Depletion and Apoptosis Respectively Microclot Formation. CPP showed maximal depletion within the first 3 minutes after induction of SAH (Figure 3(e)). A significant linear correlation was observed between CPP reduction within the first three minutes after SAH and the total number of TUNEL positive cells in the cortex (reg coeff $r = 0.73$, $r^2 = 0.53$, $P = 0.007$; Figure 4(a)) as well as in the hippocampus (reg coeff $r = 0.77$, $r^2 = 0.60$, $P = 0.003$; Figure 4(b)). However, no significant correlation was detected between relative CPP depletion within the first three minutes and the number of fibrinogen stained microvessels in the cortex (reg coeff $r = 0.42$, $r^2 = 0.17$, $P = 0.18$; Figure 4(c)) or in the hippocampus (reg coeff $r = 0.47$, $r^2 = 0.22$, $P = 0.12$; Figure 4(d)).

4. Discussion

4.1. Animal Model. In recent years, evidence has indicated that EBI and DCI largely contribute to the unfavorable outcome and mortality after aneurysmal SAH [1, 2]. Parenchymal apoptosis and microthrombosis after aneurysmal SAH are considered to be mainly involved in EBI and contributing to DCI [10–12]. Although different animal models of SAH

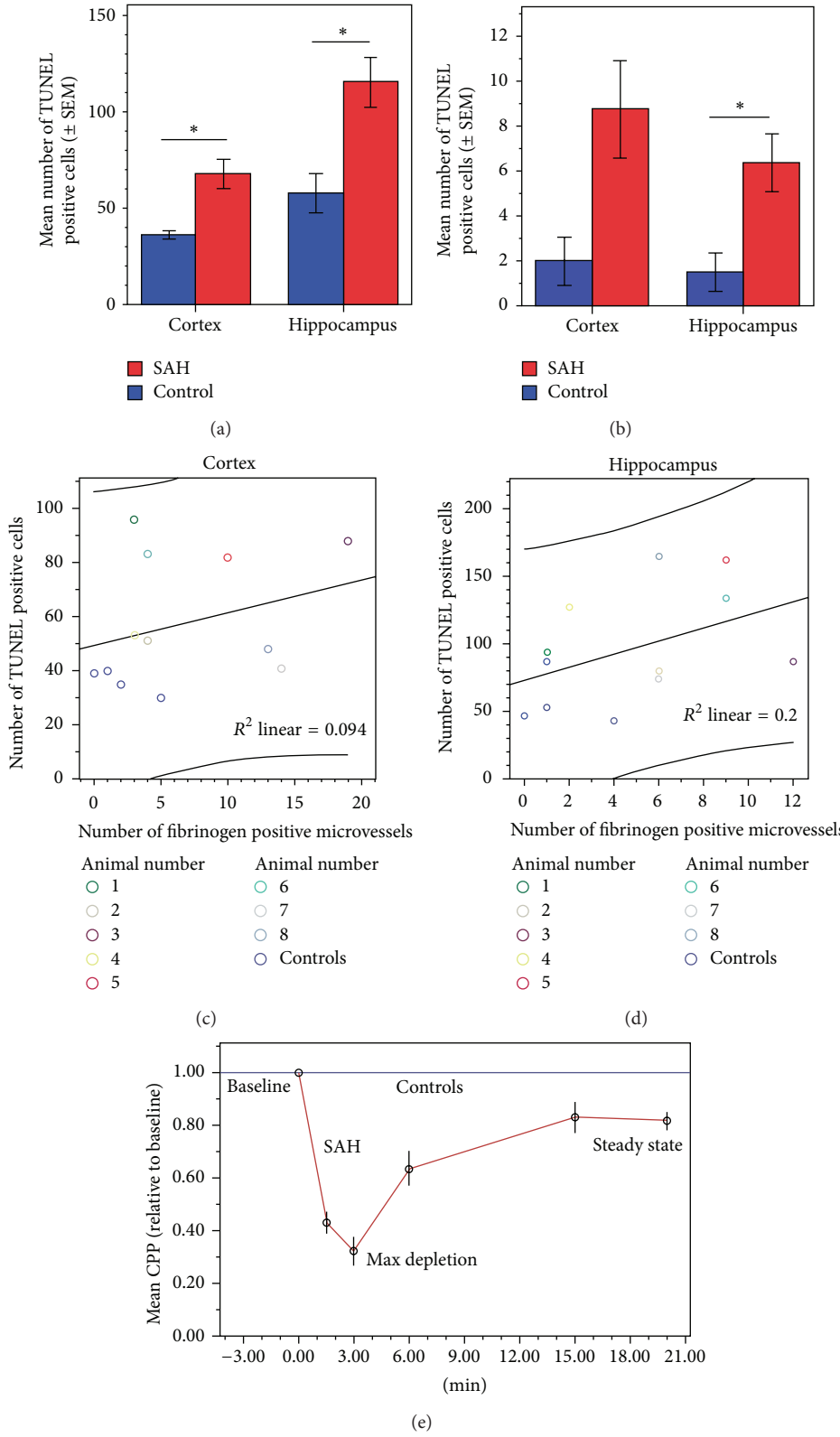

Figure 3: Evidence of microclot formation, neuronal apoptosis, and CPP depletion after experimental SAH. Quantification of TUNEL-positive cells and fibrinogen-positive stained microthrombi in cortex and hippocampus. (a) There were significant increases in the number of TUNEL-positive cells in both cerebral cortex and hippocampus as compared to controls ($P = 0.016$ and $P = 0.017$, resp., Student's t-test). (b) There was a nonstatistically significant trend towards an increase in fibrinogen-positive microvessels in the cerebral cortex ($P = 0.06$) and a statistically significant increase in fibrinogen-positive microvessels in the hippocampus ($P = 0.03$). No correlations of TUNEL-positivity with fibrinogen-positivity were observed in cortex ($R^2 = 0.094$; (c)) or in hippocampus ($R^2 = 0.2$; (d)). All data are expressed as mean ± SEM, $n = 8$ in SAH group, and $n = 4$ in control group. A P value of <0.05 was considered statistically significant. (e) Time course of the CPP depletion after induction of SAH compared to control animals.

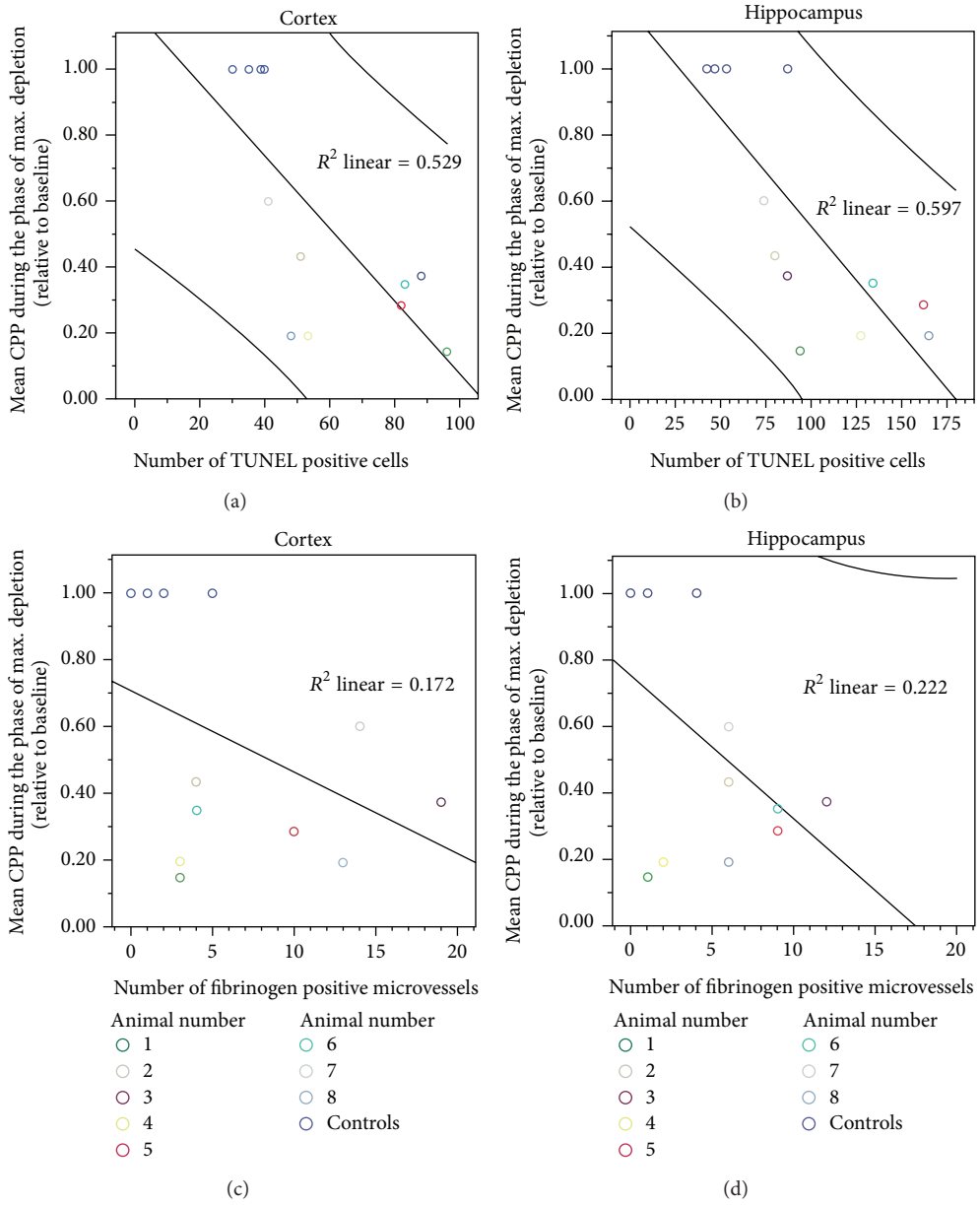

Figure 4: In the SAH group, a significant reduction in CPP was observed within the first three minutes of the phase of maximum depletion compared to the baseline or the steady state ($P < 0.001$). A significant positive linear correlation between the mean CPP during the phase of maximum depletion after induced SAH and the total number of TUNEL positive cells was found in both the cortex ($r^2 = 0.53$, $P = 0.007$; (a)) and the hippocampus ($r^2 = 0.60$, $P = 0.003$; (b)). There was no correlation between CPP depletion and fibrinogen positive microvessels in either cortex ($r^2 = 0.17$, (c)) or hippocampus ($r^2 = 0.22$, (d)). Graphs include the 95% confidence intervals.

exist [9, 13–15], it is important to investigate the impact of microthrombosis and apoptosis on EBI in animal models that represent acute pathophysiological features of SAH such as the endovascular perforation models [16–19] or ICP controlled blood prechiasmatic injection [20].

The potential important advantage of using a rabbit aneurysmal SAH model to investigate microthrombosis formation postaneurysmal SAH is the fact that the rabbit coagulation system is very similar to that in humans [21, 22]. Human-resemblance of larger animals makes them an attractive tool to provide new insights into the study of microvascular thrombosis [23, 24].

Furthermore, following aneurysm rupture, there is a rapid increase in intracranial pressure and consequent decrease in cerebral perfusion pressure [25]. Cerebral ischemia caused by increased intracranial pressure and reduced cerebral blood flow induces severe injury to the brain tissue and cerebral microvasculature [26]. To take advantage of the ability to control for ICP increase to investigate the effect on the mechanisms of cell apoptosis

and intraparenchymal microclot formation, our rabbit model closely mimics these human pathophysiological features of aneurysm rupture by arterial blood-inflow into a closed cranium [7, 8].

4.2. Microclot Formation. Recent studies detected microclots following aneurysmal SAH in humans and in models such as mice and rats [5, 19, 27–31]. In a rat model of endovascular perforation, platelet aggregates were detected in the cerebral pial microvasculature as early as 10 minutes after SAH, reaching a peak at 24 hours, and were undetectable at 48 hours [32, 33]. For the intraparenchymal microcirculation, platelets have been shown to aggregate in parenchyma microvessels within 10 minutes after SAH and persist for up to 24 hours [34].

It is still unknown whether parenchymal microvessels respond the same way as pial vessels [18, 35]. In our rabbit blood shunt model, the hippocampal brain parenchyma showed a selective vulnerability to SAH induced microthrombosis formation within 24 hours. Compared to the mouse perichiasmatic injection model used by Sabri et al. [29, 36], they observed a significant increase of microthrombosis in both the hippocampus and cerebral cortices. Although brain injury due to aneurysmal SAH causes global parenchymal damage [37, 38], vulnerability to subcortical brain regions may differ [39]. This might explain the SAH-induced significant increase of microthrombosis in the rabbit brain parenchyma at the hippocampal levels but not the cortex. Furthermore, the number of animals used in our study is small, which might explain missing correlation data in the cortex.

Finally, different mechanisms may contribute to microclot formation. An experimental model of SAH in the rat showed that a hypercoagulable state occurs immediately after injury. This abnormality in coagulation profile seemed to be a response mechanism for the acute traumatic events caused by induction of SAH in rats and may predispose them to microthrombus formation [40]. In humans, elevation of platelet activating factor and coagulation factors after SAH has been described [41]. Supporting the idea of microclot formation, markers of hypercoagulation and platelet activation increase dominantly in CSF and jugular blood compared to systemic levels after aneurysmal SAH, indicating a cerebral origin [42]. To sum up, activation of the coagulation cascade, impaired fibrinolytic activity, and inflammatory processes are particularly regarded as possible mechanisms for microclot formation [5], and different coagulation profiles among various species might be considered for future investigations regarding microclot formations.

4.3. Brain Apoptosis. Although the exact mechanism of intravascular coagulation after aneurysmal SAH is unknown, microclot formation may contribute to decreased cerebral blood flow, subsequent ischemic injury, and neuronal apoptosis supporting the development of DCI [32]. It is still a matter of debate whether the blood clot itself or the transient global ischemia after increase of the intracranial pressure is responsible for the microcirculatory changes after SAH [36]. In our rabbit model, 24 hours after SAH-induction, microclot formation paralleled by apoptotic brain cells marked by TUNEL was predominantly distributed in the hippocampus, a brain region known to be particularly vulnerable to transient ischemia [43]. This finding is consistent with several autopsy studies that detected microclots in small parenchymal vessels and demonstrated a correlation between these microclot densities and the location and severity of histological evidence of ischemia [27]. It is well know that a brief period of global brain ischemia mainly causes cell death in hippocampal subfields neurons in rodents and humans, whereas other neurons are much less vulnerable [44, 45]. Furthermore, microclot formation and neuronal apoptosis were detected in both hemispheres, indicating a more global disease after SAH than the effect of local ischemia caused by microclot formation. In MRI studies in SAH patients, delayed ischemic lesions were observed bilaterally, regardless of the site of aneurysm rupture or vasospasm [46, 47]. Similarly, microembolic signals detected with transcranial Doppler (TCD) in 40 patients with aneurysmal SAH were mostly noted bilaterally and were associated with the development of cerebral ischemic symptoms and not related to vasospasm [31]. In an endovascular perforation model in mice, positive antithrombin staining was detected bilaterally in a scattered distribution with a peak at 48 hours and then decreased gradually [30]. However, the disadvantage of this murine model using endovascular perforation is the uncertainty to determine if rebleeding occurred over the study time.

4.4. Correlation of Microthrombosis, Neuronal Injury, and CPP Depletion. CPP shortage during the hyperacute phase of SAH significantly correlated with the degree of apoptosis and neurodegeneration in the hippocampus and cortex 24 hours after experimental SAH. However, no significant correlation was found between CPP depletion and the number of fibrinogen positive microvessels in either the cortex or the hippocampus. Furthermore, there was no significant correlation between structural changes in microvessels due to early brain injury comparing TUNEL-positivity neither in the cortex nor in the hippocampus in our study findings. This is in contrast to the studies of Sabri et al. [29, 36], where there were significant correlations of microthrombosis formation and neuronal apoptosis. One possible explanation might be that an inflammatory response that accompanies neural injury has both positive and negative effects [48]. There is extensive cross talk between inflammation and coagulation, whereby not only does inflammation lead to activation of coagulation but coagulation also considerably affects inflammatory activity [49]. It is known that, within a few hours after neuronal injury to the central nervous system, numerous neutrophils induce an inflammatory reaction and express high levels of the atypical growth factor oncomodulin, a crucial factor for neuronal regeneration, and cell survival [50]. Inflammation may also cause neuroprotection via inhibition of apoptosis after ischemia [51]. However, the exact effects and mechanisms of inflammation, microthrombosis, apoptosis, and neuroprotection in the context of aneurysmal SAH remain unknown and their temporal interactions need to be analyzed in further detail.

To sum up, microthrombosis in the cerebral cortex and hippocampus in a rabbit model of SAH is an important finding by itself. To the best of our knowledge, this is the first time that early microclot formation has been demonstrated in a rabbit model of SAH. Because rabbits have a coagulation cascade very similar to that seen in humans [21, 22], these findings might provide a further piece of the puzzle in our understanding of pathophysiological aspects taking place in mammals after SAH and might contribute to successful translational studies in human trials.

5. Conclusion

This study found evidence of microclot formation and neuronal apoptosis after experimental SAH in a rabbit blood shunt model. Both microthrombosis and neuronal apoptosis may contribute to EBI and subsequent DCI in a distinct pathway. Long-term survival studies are mandatory to further analyze the impact and temporal characteristics—as well as the chronological sequence—of microthrombosis and apoptosis impact on DCI.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Authors' contribution to the study and paper preparation includes the following. Conception and design were carried out by Serge Marbacher, Javier Fandino, and Lukas Andereggen. Acquisition of data was performed by Lukas Andereggen, Serge Marbacher, and Volker Neuschmelting. Analysis of and interpretation of data were undertaken by Lukas Andereggen, Serge Marbacher, Michael von Gunten, Volker Neuschmelting, and Javier Fandino. Statistical analysis was carried out by Volker Neuschmelting, Lukas Andereggen, and Serge Marbacher. Drafting the article was done by Lukas Andereggen and Serge Marbacher. Critically revising the article was made by Serge Marbacher, Lukas Andereggen, Hans Rudolf Widmer, and Javier Fandino. Administrative, scientific, technical, and material support were provided by Hans Rudolf Widmer and Michael von Gunten. Study supervision was made by Serge Marbacher.

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Review Article

The Harmful Effects of Subarachnoid Hemorrhage on Extracerebral Organs

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Subarachnoid hemorrhage (SAH) is a devastating neurological disorder. Patients with aneurysmal SAH develop secondary complications that are important causes of morbidity and mortality. Aside from secondary neurological injuries, SAH has been associated with nonneurologic medical complications, such as neurocardiogenic injury, neurogenic pulmonary edema, hyperglycemia, and electrolyte imbalance, of which cardiac and pulmonary complications are most common. The related mechanisms include activation of the sympathetic nervous system, release of catecholamines and other hormones, and inflammatory responses. Extracerebral complications are directly related to the severity of SAH-induced brain injury and indicate the clinical outcome in patients. This review provides an overview of the extracerebral complications after SAH. We also aim to describe the manifestations, underlying mechanisms, and the effects of those extracerebral complications on outcome following SAH.

1. Introduction

The prevalence of unruptured intracranial aneurysms in health adults was found to be between 3% and 7% [1, 2]. Spontaneous rupture of intracranial aneurysms may lead to subarachnoid hemorrhage (SAH), a hemorrhagic stroke subtype with a high case fatality [1]. Nearly 30,000 individuals in the United States are affected by aneurysmal SAH each year [3]. Although early surgical or endovascular repair of ruptured aneurysms and aggressive postoperative management has improved the overall outcome in patients, SAH continues to be responsible for physical, psychological, and financial damage in developing and developed countries alike. Thus, SAH remains a worldwide leading cause of death and neurological disability. Indeed, the mortality rate is approaching 50% and less than 60% of SAH survivors return to functional independence [4, 5]. Neurologic outcome following SAH is largely determined by the amount and location of initial bleeding. Previous studies have focused on intracranial complications of SAH as independent predictors of outcome, such as early brain injury, delayed cerebral ischemia, and chronic hydrocephalus [5–9].

Aside from the primary and secondary neurological injury induced by this stroke subtype, SAH is also significantly associated with nonneurologic medical complications. Indeed, SAH patients are extremely vulnerable to multiple extracerebral organ dysfunctions (Figure 1). With improvements in the surgical and endovascular management of intracranial aneurysms, nonneurological complications will assume a more prominent role in the overall outcome of SAH patients [10], as such complications may increase the length of hospital stays as well as the need of intensive care unit management. Evidently, nonneurological organ dysfunctions correlate with the severity of brain injury following SAH. The most frequent nonneurologic medical complications occurring after SAH include pulmonary edema and pneumonia, cardiac arrhythmia, renal and hepatic dysfunction, electrolyte disturbance, and hematologic derangements [10]. Combinations of brain injury and extracerebral organ dysfunction may occur concurrently after SAH, and the latter may exacerbate

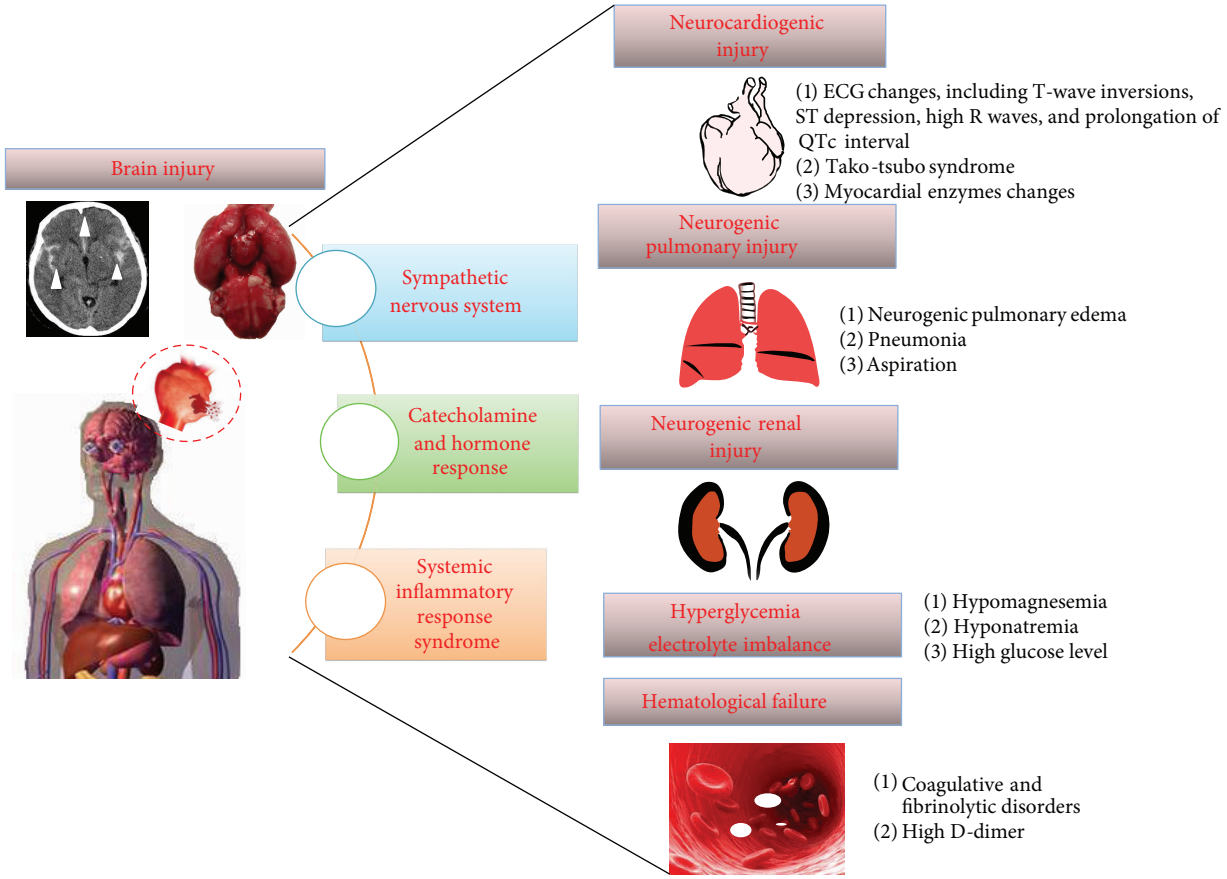

Figure 1: Schematic of nonneurologic medical complications following subarachnoid hemorrhage.

brain injury during the acute phase of bleeding. Therefore, the prevention and management of nonneurological complications are important for improving the overall clinical outcome after SAH.

This review focuses on nonneurologic complications after SAH, describing the frequency, severity, and manifestations of those complications. In particular, we discussed several underlying mechanisms of the nonneurologic complications and present treatment opportunities.

2. Possible Mechanisms of Nonneurologic Complications following SAH

2.1. Hormone Response to SAH. Psychological and physical insults to the central nervous system can trigger a disastrous response of the sympathetic nervous system, eventually leading to end-organ catecholamine-mediated injury [11, 12]. Massive sympathetic nervous activation occurs in SAH patients. Activation of the sympathetic nervous system, which leads to an elevated level of circulating, cerebrospinal fluid (CSF), and urine catecholamines, may be the link between the initial ictus and the genesis of some of the systemic complications after SAH [13]. Sympathetic activation was observed as an elevation of plasma norepinephrine following preclinical and clinical SAH studies [14, 15]. It has been well recognized that a high sympathetic tone combined with high circulating catecholamine concentrations may occur in humans with head injury, particularly after SAH [13, 16]. The amount of catecholamines released into the systemic circulation of SAH patients was found even higher than in patients with cardiac arrest or asphyxia [17]. Meanwhile, the uptake of norepinephrine was found to be decreased following SAH. Physiological derangements can occur following a sudden and sustained increase in systemic catecholamines. Catecholamines potentiate the activation of endothelin [18], which plays a role in the development of vasospasms. It has been reported that endothelin-induced cerebral vasospasms were associated with delayed cerebral ischemia following SAH [19, 20]. However, the randomized, double-blinded, placebo-controlled, phase 3 study (CONSCIOUS-2 and CONSCIOUS-3) demonstrated that the endothelin-1 receptor antagonist clazosentan decreased the occurrence of cerebral vasospasm but had no significant effect on the functional outcome after SAH [21, 22]. Catecholamine-induced stress may be associated with the well-known organ dysfunction described in SAH with the production of toxic cytokines, including high-pressure pulmonary edema, myocardial myocytolysis, stress hyperglycemia, hypokalemia, and leukocytosis (10753996, 1604280). Data from animal models and clinical studies suggest that the increased release in catecholamines is

the most likely underlying cause of cardiac injury after SAH [15, 23]. The "catecholamine hypothesis" is particularly supported by an experimental model of sudden brain death, which demonstrated immediate and massive increases in myocardial norepinephrine measured by microdialysis techniques [24]. Furthermore, hormonal profiles of SAH patients demonstrated an increase in natriuretic peptide, renin, angiotensin II accompanied with high concentration of cardiac troponin I (cTnI), and stable low levels of vasopressin. Both brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) levels in SAH patients were found to be elevated to values 2-3 times greater than those observed in healthy volunteers within 3-4 days after the ictus [25]. However, in another study, throughout the 7 days after SAH, lower than normal aldosterone concentrations and normal plasma concentrations of ANP and C-type natriuretic peptides (CNP) were found [26]. B-type natriuretic peptide showed significant diagnostic efficiency for predicting delayed cerebral ischemia after SAH [27]. Rapid natriuresis occurs prior to the development of ischemic symptoms after SAH, indicating that it is a trigger for symptomatic vasospasm [28]. Cerebral salt wasting concomitantly occurs following SAH, which induces excessive natriuresis and osmotic diuresis. Natriuresis results in the reduction of total blood volume and increases the risk of symptomatic vasospasm after SAH in patients as well as in an SAH rodent model [29, 30]. Increased levels of both ANP and BNP were found in SAH patients, which surprisingly were not related to either biomarkers or clinical severity of cardiac injury [25]. The levels of plasma natriuretic peptides were much higher than CSF levels of natriuretic peptides, which supported the view that the heart is the source of plasma ANP and BNP after SAH [25].

An approximately 3-fold increase in plasma renin activity was observed by measuring the level of angiotensin I, which indicates an acute activation of the renin-angiotensin system in the early stages following experimental SAH [31]. Significant correlation was found between urinary catecholamines excretion and both plasma renin and plasma angiotensin II concentrations. SAH patients presenting elevated plasma renin levels experienced a higher incidence of mortality and morbidity than those with lower plasma renin [32], which indicates that the renin-angiotensin system may play a role in some of the deleterious consequences of SAH [33, 34]. Angiotensin II is of importance in disruption of the blood-brain barrier and the regulation of brain capillaries permeability following SAH [35, 36]; thus it may be a link between brain injury and extracerebral organs injury. Moreover, in studies of experimental SAH, delayed cerebral vasospasm was attenuated by the treatment of an angiotensin-converting enzyme inhibitor [37]. Angiotensin receptor blockade via losartan markedly decreased the survival in experimental SAH study, suggesting that the acute activation of the renin-angiotensin system is a desirably compensatory response [31].

Taken together, hormonal changes are implicated in the pathophysiology of SAH and their influence in the pathogenesis of delayed extracerebral complications warrants further investigation.

2.2. Systemic Inflammatory Response Syndrome (SIRS). Inflammatory responses and metabolic derangements are frequently described in SAH patients [38]. These patients will often present febrile and tachycardic without underlying infections [39]. Systemic inflammatory response syndrome (SIRS) is an inflammatory phenomenon affecting the whole body, frequently a response of the immune system to infectious and noninfectious insults. SIRS accompanies various acute cerebral insults, including ischemic stroke, SAH, and intracerebral hemorrhage. SAH, as a noninfectious insult, can induce the SIRS via triggering immune system activation [40]. The surge in ICP and activation of the sympathetic nervous system contribute to SAH-induced SIRS [41]. Furthermore, patients undergoing aneurysm surgery have an increased likelihood of developing SIRS [41, 42]. Given the frequency of systemic disturbances, it has been reported that SIRS occurs with an incidence from 29% to 87% in SAH patients [43, 44]. In addition, SAH is frequently accompanied by leukocytosis, elevated levels of proinflammatory cytokines, and fever [45, 46]. Elevated levels of interleukin-6 (IL-6) and C-reactive protein (CRP) were found in the systemic circulation of SAH patients, with even higher peaks associated with delayed brain ischemia [47]. SIRS standard criteria include abnormal heart rate, respiration rate, temperature, and white blood cell count [48]. In a retrospective study, admission SIRS score proved to parallel the severity of SAH, indicated by Hunt and Hess grading, amount of clot demonstrated by radiographic examination, and plasma glucose concentration [40]. SIRS not only promoted extracerebral organ dysfunction, but also exacerbated delayed cerebral ischemia, contributing to a worsen outcome. Furthermore, SIRS contributed to acute lung injury and poor outcome after SAH [41, 49]. The admission SIRS score may be a significant outcome predictor of subsequent neurological deterioration.

3. Manifestations of Nonneurologic Complications after SAH

3.1. Neurocardiogenic Injury. Cardiac manifestations of SAH are particularly impressive, because manipulation of blood pressure parameters is routinely used as the treatment for SAH patients. In 1982, Braunwald and Kloner first defined the condition of "stunned myocardium," as a reversible postischemic myocardial dysfunction [50]. Recently, neurocardiogenic injury following SAH has been further elucidated; it includes electrocardiographic (ECG) abnormalities, arrhythmias, myocardial infarction (both non-ST elevation and ST-elevation), left ventricular (LV) dysfunction, elevation of cTnI, and even cardiac arrest [51–54]. Those conditions have been considered in relation to SAH, although their clinical relevance is still unclear [51]. Significant cardiac dysfunction or laboratory evidence of cardiac injury complicates the management of SAH patients [55]. Moreover, pathological evidence of contraction band necrosis provided evidence for the development of myocardial necrosis in heart autopsies [56]. SAH patients with cardiac injury have higher short- and long-term mortality rates [57]. Several methods are used to

identify myocardial injury, such as serial ECG, hemodynamic measurements, coronary angiography, blood flow measurements by radiolabeled microspheres, 2D echocardiography, and myocardial contrast echocardiography.

A variety of ECG changes, including T-wave inversions, ST depression, high R waves, prolongation of the corrected QT (QTc) interval, and large U waves, have been frequently documented in SAH patients, possibly because of elevated catecholamines or electrolyte imbalances. But the results of several studies demonstrated a negative relation between high levels of catecholamines and ECG changes [58, 59]. In addition, hypothalamic stimulation may induce ECG abnormalities without associated myocardial injury [60]. Furthermore, neurons of the nodose ganglia are damaged due to ischemic insult secondary to SAH. The ischemic neuronal degeneration in the nodose ganglia disturbed the afferent vagal nerve reflexes and eventually led to heart rhythm irregularities [61]. Evidence has accumulated and suggests that ECG abnormalities in the acute stage of SAH reflect a transient cardiac dysfunction rather than permanent myocardial injury. In a prospective study of 447 SAH patients, 39% of these patients experienced prolonged elevated heart rate (>95 beats/min for >12 h), which was associated with major adverse cardiopulmonary events and poor outcome after SAH [62]. Heart rate variability is a potential marker of reversible cardiac injury, severe vasospasm, and death [63–65]. In another study, 100 subjects who were admitted within 24 h after SAH demonstrated prolongation of the QTc interval. Further univariate analyses showed significant correlation between QTc interval length and other variables, such as sex, serum concentrations of potassium, calcium, or glucose. Nevertheless, these analyses suggested that only female sex and hypokalemia were an independent risk factor for severe QTc prolongation in SAH patients [66]. It has been confirmed that QTc interval prolongation improved in patients with a good prognosis; it persisted in SAH patients with a poor outcome, further indicating that a QTc interval of longer than 448 ms at 7 days after surgery can serve as a predictor of clinical outcome following SAH [67]. Twenty-three patients with SAH were examined, who showed an ST segment elevation in their ECG [68]. ECG and echocardiogram abnormalities were normalized and normalization of the apical wall motion was recorded on echocardiograms within several months after SAH, which indicated that cardiac dysfunction may be reversible. Previously, Kolin and Norris indicated that the distinctive myocardial lesion accompanying cerebral injury is reversible, because the increased level of catecholamines returned to the normal [69]. Early ECG abnormalities were associated with the in-hospital mortality of the patients with SAH, but not with the overall prognosis [70, 71].

Stress cardiomyopathy reflects merely a single aspect of a much wider range of neurocardiogenic injury, which encompasses cardiac dysfunction associated with SAH. Takotsubo syndrome is a rare acquired cardiomyopathy, characterized by LV dyskinesia and symptomatology typical of acute myocardial infarction. Although the pathogenesis of takot-subo syndrome has not yet been established, compelling literature supports the theory that acute cardiac sympathetic disruption accompanied with norepinephrine seethe and spillover are the mechanisms of tako-tsubo syndrome [72]. Historically, cardiac pathophysiology after SAH has been attributed to LV myocardial ischemia, which may be caused by coronary artery spasm and thrombosis and/or oxygen supply-demand mismatch in the setting of hypertension and tachycardia [73, 74]. Approximately 10% of all SAH patients suffer from LV systolic dysfunction [68]. A 54-year-old woman initially presented with ST elevation myocardial infarction and resultant LV failure, which was ultimately explained by the diagnosis of SAH with subsequent adrenergic storm [75]. Systolic dysfunction can be observed by echocardiography as a reduced LV ejection fraction and/or the presence of regional wall motion abnormalities of the LV. LV ejection fraction and pulse-wave velocity were related to poor outcomes following SAH [27]. A multicenter prospective cohort study found that the cardiac index was significantly lower in patients with high grade SAH (World Federation of Neurological Surgeons grades IV and V) on days 1 and 2 after the ictus [76]. This was further supported by a recent retrospective study, which highlighted the effect of norepinephrine in pathogenesis of SAH-induced wall motion abnormalities [15, 77]. In addition, postmenopausal women after poor-grade SAH are predisposed to develop wall motion abnormalities due to lack of estradiol [23]. Impaired LV hemodynamic performance was proposed to contribute to cardiovascular instability, pulmonary edema formation, and complications of cerebral ischemia [78].

Elevations in serum cardiac enzymes, including creatine kinase, MB isoenzyme (CK-MB), and cTnI, were elevated following SAH [79, 80]. Previous studies have shown that 17 to 28% of SAH patients develop elevated serum levels of cTnI [81, 82]. In severely affected patients with elevated levels of cTnI, reduction of cardiac output may increase the risk of cerebral ischemia and poor outcome related to vasospasm [81]. In a prospective study, cTnI has been shown to be a more sensitive indicator as compared to CK-MB in the detection of left ventricular dysfunction in patients with SAH [81]. A retrospective study including 617 consecutive SAH patients demonstrated that patients with high troponin levels demonstrated an increase in mortality [51]. In an SAH rat model, early activation of matrix metalloproteinases was observed in the myocardial tissue and plasma, which may enhance cTnI degradation [83]. Thus, matrix metalloproteinases antagonism may provide a protective effect against SAH-induced cardiac damage.

Interestingly, it has been reported that endovascular coiling or surgical clipping of ruptured aneurysms is not associated with the incidence of cardiac injury or dysfunction [84]. However, it is important to note that treatment decisions were made on the basis of standard practice patterns rather than on a randomization process. Intraoperative anesthetic management differs between the two procedures mentioned above. Additionally, patients who underwent aneurysm coiling were also treated with either anticoagulant or antiplatelet agents.

It is important to discover promising strategies that minimize neurocardiogenic complications. All patients with SAH require close cardiac monitoring, and, in some cases, cardiac β-adrenergic stimulation may be advisable.

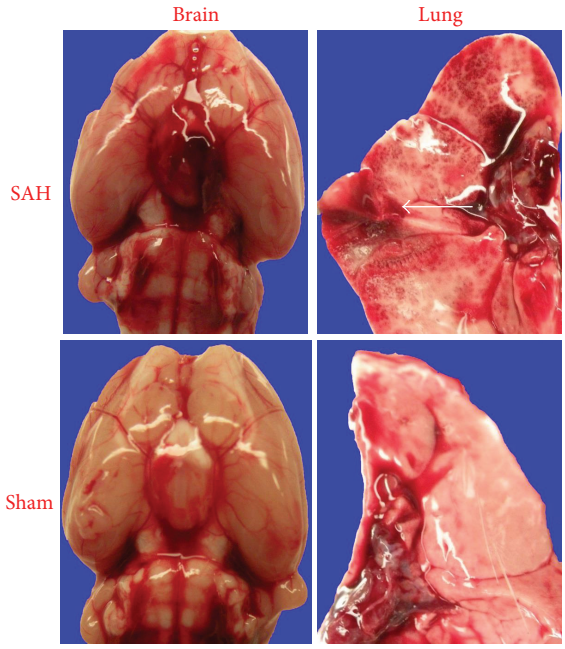

Figure 2: High-resolution pictures of subarachnoid hemorrhage and sporadic pulmonary hemorrhagic lesions in a rat endovascular puncture model (white arrow).

3.2. Neurogenic Pulmonary Edema. Pulmonary complications after SAH are a cluster of lung dysfunctions, which includes pneumonia, aspiration, and neurogenic pulmonary edema (NPE) (Figure 2) [85]. Pulmonary complications are the most frequent extracerebral cause of death after SAH [44, 86]. Oxygenation deficits occur in the acute stage of SAH. Indeed, oxygenation disturbances were found in 43% to 92% of SAH patients, which most often resulted from pulmonary edema. Patients with World Federation of Neurological Surgeons IV and V were significantly higher scored in the extravascular lung water index, pulmonary vascular permeability index, and systemic vascular resistance index on day 2 after SAH [76]. Differential diagnosis of the pulmonary complications can be difficult. NPE is usually suspected when there are no underlying lung diseases, and NPE is found in 23% to 71% patients during hospitalization [10, 87]. The incidence of pathological diagnosis of NPE is higher than its clinical diagnosis. The abnormality of NPE after SAH is often unilateral on chest X-ray. SAH patients with NPE were usually younger and died sooner than those without. The development of pulmonary edema most frequently occurs within the first week from the beginning of the SAH with a peak around day 3. The incidence of NPE decreased with time after SAH.

NPE displayed biphasic in SAH patients, first with cardiogenic NPE caused by cardiac dysfunction immediately after SAH, and hydrostatic NPE resulted from hypervolemia and low cardiac contractility 7 days after SAH [88]. NPE in SAH patients occurred for some mechanisms. First, at high pressure, disruption of the capillary endothelium and alveolar epithelium will occur due to raised capillary pressure with the development of a high-permeability of blood-lung barrier. A hydrostatic form of pulmonary edema develops. High-pressure pulmonary edema is apparently not the only mechanism. Secondly, a reversible form of cardiac injury is linked to NPE following SAH. Severe depression of left myocardial function occurring after SAH was regarded as another mechanism involved in NPE pathogenesis, as demonstrated in a retrospective study of 20 patients with NPE [89]. This is evident with most NPE patients demonstrating increased pulmonary wedge pressure and reduced cardiac output or reduced left ventricular function [78]. However, in a small sample-size retrospective study, there was no evidence for high-permeability edema or cardiac failure in half of patients who presented with oxygenation disturbances. In those patients, pulmonary edema may be due to extravascular lung water [90], because the latter was significantly and positively correlated with impaired oxygenation in a study of patients with hemorrhagic stroke [91]. Thirdly, some molecules, such as S100B, E-selectin, and caspase-1, can be the link between the brain and the lung that determine the development of NPE after SAH. S100B binds the receptor for advanced glycation end products in alveolar epithelial type I pneumocytes to amplify the immune and inflammatory response causing lung injury [92]. In an SAH mouse model, pulmonary endothelial cell apoptosis contributed to the pathophysiology of NPE [93]. Caspase-1 inhibitor can prevent the apoptosis of pulmonary endothelial cells and ameliorate NPE [94]. SAH increased the pulmonary expression of the cytokines (tumor necrosis factor-α), chemokines, and adhesion molecules (E-selectin, intercellular adhesion molecule- (ICAM-) 1, and vascular cell adhesion molecule- (VCAM-) 1). Interferon-β reduced lung inflammation following experimental SAH [95]. P2X purinoceptor 7 antagonist administration attenuates inflammation and prevents the lung-blood barrier in experimental SAH model [96]. Forth, the application of hypothermia and barbiturates in confronting high ICP may result in immune suppression, decreased leukocyte counts, and likely predisposes to pneumonia [97]. Additionally, diminished level of consciousness resulted in aspiration and impaired cough due to neurological injury. Sedation may also result in atelectasis. Furthermore, recently, vasospasm after SAH has been shown to lead to ischemic neurodegeneration in the dorsal root ganglia of the phrenic nerve, and phrenic nerve root ischemia has been suggested to play a crucial role in respiration rhythms deteriorations following experimental SAH [98]. Finally, overload of blood volume may be another contributing factor of pulmonary edema as this is generally the first intervention to maintain cerebral perfusion pressure or to ameliorate vasospasm due to aneurysmal bleeding. Recently, Mutoh et al. reported a new bedside transpulmonary thermodilution device, which is capable of distinguishing different etiologies and making fluid management decisions [99].

The majority of previous studies pay particular attention to pulmonary and cardiac dysfunction, but the burden of extracerebral organ failure after SAH, including renal, hematology, and liver, remains largely unstudied [44, 100].

3.3. Hyperglycemia and Electrolyte Imbalance. Stress hyperglycemia is present at admission in 70 to 90% of all SAH patients [101, 102]. For the mechanisms, the activation of the hepatic and pancreatic sympathetic nerve fibers resulted in increased output of glucose from the liver, a stimulation of glucagon, and an inhibition of insulin release from the pancreas [103]. Recent study suggested that catecholamine is involved in the development of hepatic insulin resistance via proinflammatory pathways [104].

Hyperglycemia exacerbates SAH-induced brain injury by enhancing the mitochondrial dynamic imbalance, apoptosis, and inflammation, which favor subsequent damage [105]. The glucose level at admission is related to the severity of initial hemorrhage [106, 107]. Previous studies revealed that the initial hyperglycemia was an independent predictor of the occurrence of delayed cerebral ischemia (DCI) and poor outcome in SAH patients. The prognostic potential of the admission plasma glucose level was suggested to be beneficial in management protocols of SAH patients [108]. Insulin therapy improved the prognosis for patients with SAH. Antihyperglycemic treatment for keeping serum glucose in normal level may be worthwhile in patients with SAH, but more preclinical and clinical studies are needed to elucidate the role of hyperglycemia in SAH.

SAH is associated with disturbances in electrolyte and circulating blood volume homeostasis. Hyponatremia occurs in 10–34% of patients who experience SAH, which worsens their prognosis [109]. Such patients exhibit excessive natriuresis [110] and resultant osmotic diuresis, which leads to a decrease in systemic blood volume [111]. All patients with SAH demonstrated increased urine output and urinary excretion of sodium [26]. Adrenomedullin, a vasorelaxant peptide, is secreted into the CSF from the choroid plexus and can exert natriuretic effects in the kidney. CSF adrenomedullin concentration was significantly higher during the late period than during the early period following SAH [112]. Results demonstrated that late-period CSF adrenomedullin concentration correlated with hyponatremia and delayed ischemic neurological deficit by logistic regression analysis. After SAH onset, hyponatremia, but not a decreased circulating blood volume, was prevented by high sodium and water infusion, adapted to renal excretion. No significant correlations were found between hormone concentrations and natriuresis. The aim of the treatment of hyponatremia is maintenance of a positive salt balance and water replacement.

Low serum potassium levels were detected in approximately 50% of all SAH patients [66]. It is believed that hypokalemia results from the catecholamine surge after SAH. High level of circulating catecholamine leads to an excessive activation of the Na+/K+-ATPase via stimulation of β2-adrenergic receptor. The consequence is a shift of potassium ions from extracellular to intracellular spaces. Thus, a lower serum potassium concentration is found in SAH patients. The effect of potassium on outcome after SAH remains controversial. It was reported that the change of potassium level was not related to outcome or DCI after SAH. On the contrary, another study showed the relationship between serum potassium on outcome and DCI [113, 114]. Thus, in cases of severe hypokalemia, potassium should be supplemented either intravenously or orally.

In addition, hypomagnesemia at admission was associated with large amounts of extravasated blood volumes, longer duration of confusion, and poor clinical condition. A multivariate analysis revealed that hypomagnesemia at onset did not predict outcome; however, hypomagnesemia can predict DCI occurring between days 2 and 12 after SAH [115]. Patients with a high serum magnesium concentration had a reduced incidence of vasospasm as examined by angiography, but the difference did not reach statistical significance [116]. Magnesium is a neuroprotective agent for inhibiting vasospasm with the rationale that its vasodilatory action on vasospastic artery and improvement of cerebral blood flow result from the inhibition of calcium channels and of myosin light chain kinase [115]. However, a retrospective analysis observed that magnesium supplementation may not reduce the incidence of symptomatic cerebral vasospasm in patients with SAH [117]. Furthermore, the conclusion of a phase 3 randomized placebo-controlled and multicenter trial was not to recommend routine administration of magnesium, because intravenous administration of magnesium sulfate was not able to improve the overall clinical outcome after SAH [118].

3.4. Renal Dysfunction. Previously, renal dysfunction has been reported in 0.8% to 7% of SAH patients [10]. The one-year mortality was significantly higher in stroke patients with kidney damage than in those without kidney damage and increased along with the progression of renal insufficiency [119]. In addition, proteinuria is an independent predictor of one-year mortality rate in patients with stroke. In a retrospective analysis of a series of 787 SAH patients, a seemingly insignificant decrease in kidney function can adversely affect the 3-month outcome independently of other known predictors [120].

Renal failure was associated with volume loading and the aggressive maintenance of mean arterial pressure. In addition, SAH-induced sympathetic activation may play a crucial role in progression of renal failure [121, 122]. SAH patients frequently receive antibiotic therapy and undergo a significant number of contrast radiographic studies, including CT angiography, CT perfusion, and catheter-based digital subtraction angiography, which have been closely associated with renal dysfunction. The combination of these factors predisposes SAH patients to acute kidney injury. However, clazosentan, a potential drug for vasospasm after SAH, was found to be well tolerated by patients with severe renal impairment and in healthy subjects, which suggests no need for adjusting the dose of clazosentan in SAH patients even with severe renal damage [123].

Herein, we highlighted the importance of close surveillance of renal function and the value of renal hygiene in the SAH. We suggested renal protection strategy for SAH patients, including avoidance of redundant contrast-enhanced imaging examination, adequate hydration and renal protection, and caution usage of potentially nephrotoxic drugs and optimal dose of those with renal impairment.

3.5. Hematological Failure. In current literature, a high incidence of coagulative and fibrinolytic disorders was observed in patients with SAH, which was also associated with outcome. Several variables of coagulation and fibrinolysis were elevated after SAH. PT, APTT, and fibrinogen were in the normal range. A prospective study showed high level of plasmatic thrombin/antithrombin complex parallels clinical outcome [124]. Specifically, a generalized elevation of plasmatic D-dimer, an index of subarachnoid clot lysis, was invariably found. Hence, D-dimer was a useful laboratory tool for assessing clinical status, since it was correlated with patients' long-term outcomes [125].

4. Prospective and Conclusion

With improvements of neurocritical care in SAH, we recommend that more attention should be shifted to nonneurological complications. First, animal model of SAH that mimics the pathophysiology after SAH will be an invaluable tool. The limitations of recent models must be carefully considered. First, the nature of aneurysm rupture is sudden and unpredictable; however, there are no naturally occurring animal models of SAH. Generally, SAH animal models used two major techniques to simulate SAH: an injection model and a vascular perforation model. The injection model neglects the importance of the injury to the artery in the pathophysiology of SAH, has high risk of mechanical damage to brain tissues, and requires craniotomy. The drawbacks of endovascular puncture model are large variations in the severity of bleeding and a high mortality rate. Besides, Wada et al. established a mouse model of intracranial aneurysm that the rupture of aneurysms would occur within a predictable time course [126]; nevertheless, it requires more experiments. Secondly, various species of SAH models are different in genome, anatomy, and physiology from humans. In addition, young animals without other diseases are used, but SAH patients often present with other diseases, such as hypertension, diabetes, and cardiopathy. At last, each model has its priority to study certain aspects of the pathophysiological process behind SAH. Future studies should differentiate suitable SAH models that target nonneurological complications. Secondly, the mechanisms of those nonneurological complications after SAH need further study. Although sudden increase in cardiac sympathetic nervous activation was believed to be the most important mechanism, it is seemingly difficult to measure it in humans [127]. It only relies on indirect method by measuring the level of circulating catecholamines. Further studies are needed to explore this issue in SAH patients. Thirdly, most current results come from retrospective analyses, which have many methodological shortcomings of purely retrospective studies. Thus, it is extremely important to execute large, double-blind, randomized, prospective trials evaluating the frequency, severity, role, and therapeutic strategy of nonneurological medical complications after the rupture of aneurysms [128, 129]. Furthermore, severity of illness scores is frequently used for daily assessment in SAH patients, such as Sequential Organ Failure Assessment (SOFA) score and APACHE II score [130, 131]. The efficiency of those scores requires validation in SAH populations. Recently, for patients with SAH, treatments commonly involve the management of intracranial hypertension and the support of cerebral perfusion pressure with volume loading and inotropes. However, cerebral perfusion pressure-targeted management of intracranial hypertension in SAH patients may lead to nonneurological complication (e.g., NPE) and eventually worsen outcome [132]. Thus, we stressed that potential adverse effects of currently management strategy may offset their beneficial effects. We suggest developing a more efficient treatment strategy before it is too late. At last, we emphasized that the physician should keep the nonneurological complication after SAH in mind. For example, if a patient was diagnosed as having acute myocardial infarction with ECG changes and troponin elevation, who also presented neurologic symptoms or signs, brain computed tomography should be performed to exclude SAH before the thrombolytic therapy.

5. Conclusion

SAH is not only affecting brain tissue, but also impairing extracerebral organs. Extracerebral complications are associated with the high mortality rates and neurological impairments following SAH, even after adjustment for the severity of the initial neurological injury. All of the nonneurologic complications have been linked to adverse clinical outcomes, such as circulatory failure, NPE, electrolyte imbalance, or hyperglycemia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Inflammation, Vasospasm, and Brain Injury after Subarachnoid Hemorrhage

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Subarachnoid hemorrhage (SAH) can lead to devastating neurological outcomes, and there are few pharmacologic treatments available for treating this condition. Both animal and human studies provide evidence of inflammation being a driving force behind the pathology of SAH, leading to both direct brain injury and vasospasm, which in turn leads to ischemic brain injury. Several inflammatory mediators that are elevated after SAH have been studied in detail. While there is promising data indicating that blocking these factors might benefit patients after SAH, there has been little success in clinical trials. One of the key factors that complicates clinical trials of SAH is the variability of the initial injury and subsequent inflammatory response. It is likely that both genetic and environmental factors contribute to the variability of patients' post-SAH inflammatory response and that this confounds trials of anti-inflammatory therapies. Additionally, systemic inflammation from other conditions that affect patients with SAH could contribute to brain injury and vasospasm after SAH. Continuing work on biomarkers of inflammation after SAH may lead to development of patient-specific anti-inflammatory therapies to improve outcome after SAH.

1. Introduction

Subarachnoid hemorrhage (SAH) remains a devastating disease, leaving survivors with neurological injuries that range from subtle cognitive deficits to disabling cerebral infarctions. While treatment continues to evolve and improve, there are few therapies that treat the underlying pathological mechanisms of SAH. Additionally, there is no clear explanation for the heterogeneity among patients with SAH, with some recovering well and others worsening after their initial ictus. In this review, we will discuss the evidence supporting the role of inflammation as a direct mediator of neurological injury after SAH and a causative factor of post-SAH vasospasm. We hypothesize that the diffuse inflammatory response after SAH results in acute and chronic neurological injury and vasospasm and that patients with more severe inflammatory responses may experience worse outcomes after SAH. An improved understanding of the inflammatory pathways activated after SAH will likely lead to novel therapies and improved patient outcomes.

2. Evidence for Acute Inflammation after Subarachnoid Hemorrhage

2.1. Detection of Inflammatory Mediators in CSF after SAH. Several human studies have repeatedly shown elevated inflammatory mediators within CSF after SAH. While the key mediators identified may vary across studies, the relationship between elevation, onset of vasospasm, and decreased neurological outcomes remains a consistent finding [1, 2]. A study by Polin and colleagues [3] showed that patients who developed vasospasm after SAH had higher CSF levels of E-selectin, an endothelial cell molecule that induces leukocyte adherence and extravasation and subsequent tissue injury in ischemic stroke [4–6]. These findings are supported by

experimental data showing that CSF from patients with SAH increased rolling and adhesion of leukocytes in an *in vitro* mouse model [7]. While there are other studies showing elevation of E-selectin after SAH [8], some have failed to detect E-selectin in the CSF of patients with SAH, even when other inflammatory molecules, such as monocyte chemoattractant protein-1 (MCP-1), were elevated [9]. Other cytokines that have been widely cited to play a role in SAH, such as tumor necrosis factor-alpha (TNFα), also show wide variation in expression when compared across different studies. For instance, Kikuchi and colleagues found elevated levels of interleukin- (IL-) 6 and IL-8 but not TNFα after SAH, while other groups have found elevations of TNFα in the CSF after SAH [2, 10, 11]. One recent study found detectable levels of TNFα in only 30% of patients after SAH, indicating that the inflammatory response after SAH may be quite heterogeneous [12]. The differences across these studies may be the result of different CSF collection times after SAH, alternative methods of detection used, or diverse patient populations. In addition, cross contamination of CSF with blood during collection from ventricular or lumbar sources is rarely accounted for. The volume of blood present within the subarachnoid space would obviously affect the levels of cytokines present in CSF, and therefore cytokine concentration in CSF may reflect the volume of SAH rather than the magnitude of the inflammatory response within the brain.

One of the most widely studied molecules in SAH is endothelin-1 (ET-1), a vasoconstrictor produced by endothelial cells. ET-1 has been detected in CSF from patients with SAH and can be produced by monocytes isolated from CSF of SAH patients [13, 14]. ET-1 has been implicated in the development of vasospasm after SAH [15] and will be discussed in detail later in this review. As with many other proinflammatory molecules, the expression of ET-1 is highly variable: in a study by Fassbender and colleagues, ET-1 was not found in CSF of control subjects, and only 46% of patients with SAH had detectable levels of ET-1 [13]. Though the averaged results of both groups revealed a significant increase in ET-1 after SAH, this demonstrates that not all patients with SAH experience the same inflammatory response. Furthermore, a study from a different group failed to detect ET-1 after SAH [16]. This heterogeneity is readily apparent to clinicians treating SAH, as many patients move through their posthemorrhage course with few complications, while others experience severe complications such as vasospasm and cerebral edema, which may both be driven by an inflammatory response [17, 18].

2.2. Detection of Inflammatory Mediators in Blood after SAH. In addition to the inflammatory cytokines found within CSF in patients after SAH, a systemic increase in inflammatory mediators after SAH is well documented [1, 19, 20]. This systemic increase in inflammatory cytokines after SAH is predictive of poor outcome and may be related to a late, rather than early, inflammatory response [21–24]. Other markers of systemic inflammation, such as high body temperature and leukocytosis, have also correlated with worse outcomes after SAH; however, no causal relationship was established between peripheral inflammation and intracerebral pathology [25, 26]. There is increasing interest in developing inflammatory biomarkers for prognosticating outcome in SAH though no biomarker study in SAH has been prospectively validated [27]. It is unclear to what extent the presence of inflammatory cytokines in plasma is due to intracerebral processes or how strongly blood cytokine levels correlate with inflammation within the brain [12]. It is quite possible that inflammatory cytokines detected in plasma are indicators of global inflammation throughout the body, as would be expected in patients who are critically ill after SAH. For example, both ICAM-1 and E-selectin have been used to prognosticate outcome in critically ill patients *without* SAH [28]. It is also possible that inflammatory cytokines within the blood contribute to brain injury after SAH; the breakdown of the blood brain barrier after SAH could allow serum cytokines to enter the brain parenchyma leading to tissue injury there.

In planning any trial of anti-inflammatory therapy after SAH, consideration should be given to when the peak of inflammation occurs, in order to optimize delivery and dosing of the therapy. There is evidence that the expression of certain inflammatory molecules peaks within 24 hours after hemorrhage onset, with IL-6 being elevated at days 0 to 1 after SAH [29] though others have shown a peak of IL-6 at day 6 after SAH [30]. Once again, differences between these two studies may be due to many factors, including the high degree of variability among inflammatory responses among patients with SAH [3, 10].

2.3. Inflammation and Aneurysm Formation. Inflammation may also play a role in aneurysm formation. Several previously discussed inflammatory cytokines, such as TNFα and MCP-1, are thought to play a role in endothelial injury and remodeling based on evidence from both human and animal studies [31, 32]. A recent clinical study found increased cyclooxygenase within the walls of ruptured and unruptured aneurysms [33] and the same group has shown that aspirin may reduce the rate of aneurysm rupture due to its anti-inflammatory properties [34, 35]. E-selectin has also been found at elevated levels in the walls of aneurysms [36]. Additionally, environmental factors that contribute to a proinflammatory state may contribute to aneurysm formation [37]. A detailed discussion of the contribution of inflammation to aneurysm formation is outside the scope of this review and was reviewed recently elsewhere [38].

3. Evidence for Inflammation in Animal Models of SAH

Animal models have been utilized to establish a causative relationship between inflammation and brain injury after SAH. Several animal models for SAH exist, each with their own advantages and drawbacks [39]. Common models include blood injection into the basal cisterns of animals or endovascular perforation, both of which produce vasospasm and an inflammatory response [40–42]. As in human SAH

studies, there is considerable variability between subjects when inflammatory responses are quantified [43, 44]. Animal studies of SAH have found evidence of inflammation in all intracerebral compartments: CSF, brain parenchyma, and vasculature [45–49].

Animal studies have been used to link inflammation, cerebral edema, and cell death after SAH. The anesthetic isoflurane has been shown to reduce TNFα production, leukocyte adhesion molecule expression, and blood brain barrier permeability after experimental SAH [50, 51]. The results of these studies are promising but do not prove whether decreased TNFα levels in affected tissue are a cause or result of decreased leukocyte infiltration. There is, however, evidence of TNFα having a direct role on neuronal injury after SAH. Recently, blockade of TNFα was shown to reduce apoptosis in the hippocampus after SAH [52]. This corresponds well to the extensive literature demonstrating a role for TNFα in neuronal apoptosis in other forms of neurological injury [53]. However, the antiapoptotic effects of TNFα blockade were not uniform throughout the brain, and a pre-rather than posttreatment paradigm was utilized in this study [52]. Furthermore, there is not even a consensus that neuronal death occurs in SAH, with some groups reporting no neuronal apoptosis after SAH while others observe neuronal death throughout the brain after SAH [54–56]. Regardless of whether or not neuronal death occurs after SAH, there are other pathological events that could explain neurological dysfunction after SAH, such as synaptic injury, loss of long term potentiation, and white matter injury [57]. Both synaptic loss and white matter injury are mediated by inflammation in models of other neurological diseases; however, more work is needed to understand the precise role of inflammation on cell death and injury after SAH [58, 59].

Animal models of SAH have also found evidence for inflammation playing a role in injury outside of the brain. In a study using endovascular perforation as a model of SAH in rats, systemic anti-inflammatory treatment was able to reduce lung injury after SAH [60]. This is relevant to SAH treatment as many patients experience cardiopulmonary complications as part of a systemic reaction to SAH [61]. Animal models of SAH have played an essential role in linking inflammation to vasospasm after SAH and will be discussed in more detail below.

4. The Role of Inflammation in Vasospasm after SAH

4.1. Induction of Vasospasm with Proinflammatory Agents. Early clinical studies showing a correlation between vasospasm and fever in the absence of infection established a link between inflammation and vasospasm [77–86]. Several proinflammatory agents such as talc (crystallized hydrous magnesium sulfate) [87, 88], latex, polystyrene, and dextran beads [89, 90], lipopolysaccharide (LPS) [91], and tenascin-C [92] have been administered intracisternally to show that vasospasm can occur in the absence of blood. These studies provided proof that vasospasm is not dependent on red blood cells (RBCs) or hemoglobin (Hgb) and confirmed the role of inflammation in the development of vasospasm.

4.2. Inflammatory Molecules Linked to Development of Vasospasm. Among inflammatory molecules linked to cerebral vasospasm, the selectin family, which consists of three members: E-selectin, platelet- (P-) selectin, and leukocyte- (L-) selectin, has been extensively studied. These molecules facilitate leukocyte binding and migration through vascular endothelium towards injured tissue. L-selectin and E-selectin are constitutively expressed on cell surfaces whereas P-selectin expression requires activation by histamine or thrombin [93]. E-selectin is elevated in the CSF of SAH patients with higher concentrations seen in patients who develop moderate or severe vasospasm [3]. Inhibition of E-selectin with an inhibitory antibody [62] and E-selectin tolerization via intranasal administration have decreased vasospasm in rodent SAH models [63]. However, not all data point to selectins having deleterious effects after SAH: while P-selectin levels were higher in patients with SAH who developed cerebral ischemia after SAH, L-selectins were higher in patients who did not develop delayed ischemia [23, 94].

Integrins are cell surface proteins that facilitate cell-cell adhesion and interaction. The main integrins involved in leukocyte adhesion and migration are lymphocyte function-associated antigen 1 (LFA-1) and Mac-1 integrin (CD11b/CD18). Systemically administered anti-LFA-1 and Mac-1 monoclonal antibodies reduce vasospasm in rat [64], rabbit [65], and primate [66] SAH models. Immunoglobulin superfamily proteins, such as ICAM-1, play a role in leukocyte adhesion and are upregulated in patients who develop clinical vasospasm [3] as well as in rabbit [70] and canine SAH models [47]. Anti-ICAM-1 monoclonal antibodies were shown to decrease femoral artery vasospasm and inhibit infiltration of macrophages and neutrophils into blood vessel adventitia in a rodent model [95] and reduce vasospasm in a rabbit model of SAH [71].

Key proinflammatory cytokines elevated in experimental models and patients with vasospasm include IL-1B, IL-6, IL-8, TNFα, and MCP-1. IL-6 has been shown to peak early after SAH, suggesting that it may be an early marker for predicting vasospasm development [9, 11, 47, 96–101]. TNFα levels in poor-grade SAH patients were shown to correlate with severity of vasospasm [68] and serum MCP-1 levels were associated with predicting negative outcome but not severity of vasospasm [21]. Cytokine inhibitor CNI-1493 [72], anti-IL-6 antibodies [102], anti-IL-1B antibodies [67], and TNFα inhibitors [103] have all been shown to attenuate vasospasm in animal models.

Several studies have examined intracellular signaling pathways activated during inflammation and their role in vasospasm. Mitogen-activated protein-kinase (MAPK) and nuclear factor kappa-B (N$f\kappa$B) intracellular signaling pathways are crucial in generating inflammatory immune responses [104]. Jun N-terminal kinase 1 (JNK1) and JNK2, which are part of MAPK family, are activated in the cerebral vasculature after experimental SAH and their inhibition reduces vasospasm [105, 106]. Inhibition of JNK was also effective at reversing the vasoconstrictive effects of tenascin-C in a rat model of SAH [92]. Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that regulates adhesion molecule

expression and neutrophil recruitment during inflammation [107]. In a rabbit model of SAH, Satoh and colleagues showed PARP activation within the smooth muscle and adventitia of blood-exposed vessels and that PARP inhibition decreased the severity of vasospasm [108].

The complement pathway of antibacterial proteins also affects vasospasm after SAH. Complement depletion by treatment with cobra venom [109] and prevention of complement activation with nafamostat mesilate, a serine protease inhibitor, reduced vasospasm in experimental models [90, 110] and human subjects [111, 112]. Moreover, expression of the membrane attack complex (MAC) is increased in a rat model of SAH and can be responsible for lysis of extravasated erythrocytes and release of hemoglobin after SAH [113]. Recently, the lectin complement pathway (LCP) has also shown to be activated after SAH, and LCP activity has been linked to SAH severity and vasospasm in humans [114].

Oxidative signaling and oxidative stress are effectors of the immune response in many central nervous system diseases [115], and it is likely that the balance of oxidative stress and antioxidants influences response to and recovery from SAH. Haptoglobin is a serum protein composed of tetramer of two α and two β chains. Its main action is to bind free hemoglobin and facilitate its uptake and clearance. This has a net effect of reducing oxidative stress caused by free hemoglobin [116, 117]. Three phenotypes of haptoglobin (Hp) have been identified in humans: Hp 1-1, Hp 2-1, and Hp 2-2 [118]. In humans, the haptoglobin proteins with α-2 subunits have been associated with higher rates of vasospasm compared to haptoglobin α1-α1 [119]. Similarly, genetically modified Hp 2-2 mice experience increased macrophage infiltration in the subarachnoid space, more severe vasospasm, and worse functional outcome after SAH [120]. Recently, Hp 2-2 phenotype was associated with neurological deterioration but not cerebral infarction or unfavorable outcome in one clinical SAH study [121]; however, another recent study did find worse clinical outcomes in patients with the 2-2 phenotype [122]. Ongoing work in this area will further clarify the role of haptoglobin phenotype in SAH.

Endothelium-derived relaxing factor or nitric oxide (NO) is synthesized enzymatically by three main nitric oxide synthase (NOS) isoforms, endothelial (eNOS), neuronal (nNOS), and macrophage inducible NOS (iNOS) [123]. Under physiologic conditions, NO affects signaling pathways for vasodilation and cytoprotection among many others [123, 124]. The function of eNOS can be altered in many disease states such as atherosclerosis, hypertension, and diabetes mellitus, in which case eNOS starts to produce superoxide anion (O_2^-) instead of NO, an alteration defined as "eNOS uncoupling" [125]. Increased eNOS and iNOS expression were detected in mice after SAH, and this physiological response to SAH is decreased in proinflammatory Hp 2-2 transgenic mice compared with Hp 1-1 mice [126, 127]. In an animal model of SAH, simvastatin was shown to recouple eNOS and improve outcome after SAH [128]. On the other hand, genetic elimination of eNOS in knockout mice reduces the incidence of vasospasm and reduces oxidative stress as measured by superoxide production but has no effect on iNOS [129]. eNOS knockout mice also exhibit reduced Zn^{2+} release after SAH, which was associated with reduced microthrombi formation and neuronal degeneration in the same experiment.

Endothelins, which are potent vasoconstrictors and proinflammatory mediators expressed by vascular endothelial cells and vascular smooth muscle cells [130], are thought to contribute to tissue inflammation and cerebral edema. Several studies have documented increased ET-1 levels in SAH patients with symptomatic vasospasm and that the amount of blood within the cisterns correlated with the level of ET-1 in CSF [73, 76, 131]. However, other studies have failed to find significant elevation of ET-1 after SAH or a correlation between ET-1 levels and vasospasm [16, 73]. Inhibition of ET-1 by administration of anti-ET-1 monoclonal antibodies [132], anti-ET receptor antibodies [74, 133], ET activation enzyme inhibitors [134], and levosimendan (which antagonizes the ET receptor) [135] was effective in decreasing vasospasm in some [74, 133] but not all studies [135, 136]. Transgenic mice overexpressing ET-1 experienced more pronounced vasospasm and cerebral edema and an ET-A receptor antagonist decreased vasospasm and edema in the same study [137].

4.3. Trials of Anti-Inflammatory Agents for Treatment of Vasospasm. As subarachnoid blood is thought to generate much of the acute inflammation in SAH, faster clearance of subarachnoid clot could potentially improve outcomes after SAH. This theory has been tested, and intrathecal administration of thrombolytic agents has decreased vasospasm and improved outcomes in primates [138] and humans [139–143] after SAH. A meta-analysis showed a beneficial effect of thrombolytic treatment, with absolute risk reductions of 14.4%; however, the results of the analysis were limited due to predominance of nonrandomized studies [144]. In a recent clinical study by Yamamoto et al., cisternal irrigation therapy using tissue plasminogen activator in patients who underwent surgical clipping of ruptured intracranial aneurysms decreased serum inflammatory markers, reduced the incidence of ischemic lesions, and was associated with better neurological outcome [145]. Kim and colleagues recently demonstrated that cisternal irrigation with papaverine, a phosphodiesterase inhibitor and potent vasodilator, was similar in effectiveness compared to the thrombolytic urokinase, both of which decreased incidence of vasospasm [146].

Corticosteroids are potent anti-inflammatories and have been used in several human SAH trials. Experimental administration of high-dose methylprednisolone has been shown to reduce angiographic vasospasm and ameliorate arterial wall abnormalities in dog models [147–149]. Human clinical studies by Chyatte and colleagues [150] showed that high-dose methylprednisolone treatment improved neurological outcomes, reduced mortality, and delayed cerebral ischemia. A multicenter study of intravenous hydrocortisone administration after SAH showed improved mental status, speech, and motor function at 1 month after treatment [151]. However, another randomized controlled trial of hydrocortisone did not show any effect on neurological outcome after SAH [152]. On the other hand, hydrocortisone has been shown to support hypervolemic therapy by attenuating natriuresis

[153], inducing hypervolemia, and improving the efficiency of hypervolemic therapy [154]. However, a large double blind randomized control trial demonstrated a beneficial effect of methylprednisolone in functional outcome one year after SAH but no effect on vasospasm [155]. These studies underscore the fact that outcomes in SAH are not solely dependent upon the development of vasospasm.

Nonsteroidal anti-inflammatory drugs (NSAIDs) also have potent anti-inflammatory properties, mediated in part by inhibition of cyclooxygenase expression, which reduces prostaglandin synthesis [156]. NSAID administration significantly reduced the severity of vasospasm in an animal model of SAH; however, the mode of NSAID treatment in this model, 30 minutes before and 3 hours after SAH, is not applicable to human trials [157]. Ibuprofen inhibits femoral artery vasospasm and decreases periadventitial monocytes and macrophages after, in a rodent model of SAH, and can increase cerebrovascular diameter in monkeys and rabbits [158–160]. Chyatte and colleagues also demonstrated that ibuprofen prevented ultrastructural changes in the cerebral vessel walls of dogs after blood injection [149]. The nonsteroidal anti-inflammatory drugs parecoxib and celecoxib have also shown promise as treatment options for vasospasm [161–163]. As these drugs are relatively safe and well studied in humans, they hold promise for clinically applicable treatment options for vasospasm.

Immunosuppressants such as cyclosporine cause T-cell dysfunction by inhibiting interleukin-2 (IL-2) transcription [164] and have been tested in experimental SAH with varied success [165, 166]. Clinical studies with cyclosporine are also varied, showing no beneficial effect of cyclosporine in the outcome patients with severe SAH [167], but showing improved outcome in patients who underwent early clipping after SAH when combined with nimodipine [168–170]. Tacrolimus (FK-506), a newer immunosuppressant, did not prevent vasoconstriction and lymphocytic infiltrations in experimental SAH models [171–173].

Statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors clinically used as cholesterol-reducing agents. Their ability to reduce the expression of proinflammatory cytokines and inhibit leukocyte integrins confers their potent anti-inflammatory activity [174, 175]. Preconditioning of rats with atorvastatin has been shown to reduce the level of ET-1 while attenuating vasospasm, which could be a mechanism of antivasospastic effects of statins after SAH [176]. Simvastatin treatment before or after SAH was also shown to attenuate cerebral vasospasm and neurological deficits, possibly via endothelial nitric oxide upregulation [177], and decrease perivascular granulocyte migration [40]. Several clinical studies have shown that statins decrease serum ICAM-1 levels in hypercholesterolemic patients [174, 175, 178–180], which could explain the experimental findings associated with decreased leukocyte migration. However, clinical studies with statins have yielded mixed results. While one study showed improved 14-day functional outcomes and cerebral vasospasm in patients receiving statins prior to their SAH [181], more recent studies [182, 183] did not find significant differences in the severity of angiographic or clinical vasospasm or neurologic outcomes of patients receiving statins after SAH. Another case-control study showed that oral atorvastatin treatment decreased vasospasm and cerebral ischemia but did not lead to significant functional improvement 1 year after SAH [184]. In a phase-II randomized controlled trial enrolling 80 patients with SAH, patients treated with oral pravastatin 72 hours after SAH had a 32% reduction in vasospasm incidence, and vasospasm-related neurologic deficits and mortality were decreased by 83% and 75%, respectively [185]. Subsequently, pravastatin was also effective at sustaining the improved neurological outcome at 6 months after the treatment [186]. A Cochrane review of clinical trials on statins after SAH concluded that, in the only clinical trial that met criteria, although simvastatin improved vasospasm, mortality, and functional outcome, these benefits were not statistically significant [187]. Currently, clinical trials including simvastatin in aneurysmal subarachnoid hemorrhage (STASH) trial are ongoing (http://clinicaltrials.gov/show/NCT00731627).

Nitric oxide (NO) depletion contributes to the pathogenesis of cerebral vasospasm after SAH [188, 189]. Therefore, several NO donors have been investigated for treatment of vasospasm. Intrathecal NO supplementation via controlled-released polymers was shown to prevent vasospasm in rat and rabbit models of SAH [190, 191] and delayed polymer implantation 24 or 48 hours after SAH has been shown to be still effective at ameliorating vasospasm [191]. Several other studies have also shown that selective intracerebral NO injection, [192] intraventricular NO injection [193], and systemic nitrite infusions can improve the severity or decrease the incidence of vasospasm in experimental and clinical studies [189]. Intravenous sodium nitrate ($NaNO_2$) was also shown to reduce the degree of vasospasm and nitrite, nitrate, and S-nitrosothiols concentrations in CSF were found to be increased compared to controls in primate model of SAH [194]. L-citrulline is an amino acid that when converted to L-arginine increases nitric oxide (NO) production by NO synthase (NOS), leading to vasodilation [195]. L-citrulline administration has been shown to prevent posthemorrhagic cerebral vasospasm in the transgenic Hp 2-2 model of SAH, improve neurological function as determined by PGA (posture, grooming, and ambulation) scores, and reverse the decrease in upregulation of iNOS and eNOS expression in Hp 2-2 animals compared with baseline levels in mice [126]. Besides vasodilation, NO supplementation can have anti-inflammatory effects through modulating leukocyte-endothelial cell interactions in the acute inflammatory response. Inhibitors of NO production increase leukocyte adherence [196], and NO modulates oxidative stress [197] and microvascular permeability [198, 199]. The anti-inflammatory effects of NO through prevention of leukocyte adhesion have been linked with its ability to inactivate the superoxide anion [200]. Besides ameliorating vasospasm, whether NO donors including citrulline can help recoupling of eNOS, decrease the inflammatory infiltration, and decrease neuronal apoptosis requires further investigation. Other NO donors such as sodium nitroprusside and nitroglycerin are not considered as potential candidates due to their side effects such as dose-limiting hypotension, cyanide toxicity, and tolerance development [201].

Clazosentan, a synthetic endothelin receptor antagonist (ETRA), has been investigated as a potential treatment for vasospasm after subarachnoid hemorrhage [202]. In the CONSCIOUS-1 trial, an intravenous infusion of clazosentan 5 mg/h decreased vasospasm but the study was not powered to detect changes in morbidity and mortality [203]. In CONSCIOUS-2, a phase-III randomized controlled trial, including 1,157 patients, clazosentan infusion up to 14 days after hemorrhage did not reduce vasospasm-related morbidity and mortality or improve functional outcome [75]. A meta-analysis of randomized controlled trials for ETRAs for the treatment of vasospasm, including 5 trials with 2601 patients, showed that ETRAs decreased incidence of angiographic vasospasm; however, they did not improve functional outcome, vasospasm-related cerebral infarction, or mortality [204]. As a result, the use of ETRAs in patients with SAH was not proven to be beneficial [204]. These studies reinforce that vasospasm alone cannot be accounted for the neurological deficits and functional outcome after SAH and treatment strategies that only target improving or preventing vasospasm are not likely to succeed.

Cilostazol is a selective phosphodiesterase III inhibitor that is used to treat ischemic peripheral vascular disease and exhibits anti-inflammatory properties including inhibiting microglial activation [205, 206]. Oral cilostazol administration prevented vasospasm in a rat model of SAH [207] and reduced endothelial damage in a canine model of SAH [208]. Clinical studies have demonstrated effectiveness of cilostazol in decreasing incidence and severity of vasospasm [209, 210]. A multicenter randomized clinical trial of cilostazol has shown a decrease in angiographic vasospasm but no improvement in outcomes 6 months after SAH [211].

5. The Role of Inflammation in Early Brain Injury and Delayed Neurological Deterioration after SAH

While there is no doubt that inflammation occurs after SAH, a link must be made between inflammation and poor outcomes after SAH for it to be considered an important therapeutic target. As demonstrated by several of the trials discussed above, vasospasm is not the only determinant of outcome after SAH. It is likely that inflammation plays multiple roles after SAH, mediating vasospasm as tissue damage as well as leading to regeneration or recovery as has been shown in other neurological conditions [212]. Alternately, the inflammatory cytokines detected in the CSF and blood after SAH could be results, rather than causative factors, of brain injury after SAH. There is a vast literature in ischemic stroke that ties the inflammatory response to deleterious effects such as edema and neuronal loss. Many experimental studies in stroke have shown blood brain barrier breakdown to be mediated by inflammatory cytokines [213] and that inhibiting inflammation reduces cerebral edema and neurological injury [214, 215]. Proinflammatory cytokines such as TNFα, IL-1, IL-6, and leukocyte adhesion molecules have been associated with worse outcomes in ischemic stroke [216]. However, despite the vast literature on inflammation in ischemic stroke, there have been no successful clinical trials using anti-inflammatory agents.

Recently, the term "early brain injury" has been used to describe the mechanisms of acute neurologic deterioration after SAH [217]. Early brain injury includes cell death, cerebral edema, and neuronal dysfunction that occur after SAH. Although vasospasm is a major cause of clinical deterioration after SAH, recent thinking has focused on the fact that vasospasm is not the only phenomenon contributing to poor patient outcomes after SAH [218, 219]. A key example of this is that nimodipine, the only pharmaceutical treatment shown to improve outcomes in SAH, appears to manifest its effects without affecting the rate of vasospasm [220, 221]. Many patients also undergo neurological deterioration in a delayed fashion after SAH. This can be due to cerebral vasospasm, which typically peaks 7 to 14 days after SAH, but also occurs in the absence of vasospasm. This subacute neurological decline is referred to as "delayed neurological deterioration" and can be mediated by several events including delayed ischemia (with or without vasospasm), seizures, and fevers [222]. The terms "delayed neurological deterioration (DND)" and "delayed cerebral ischemia (DCI)" are often used interchangeably, but it should be noted that DCI is just one of the causes of DND [223]. Additionally, it is likely that the events of early brain injury occur along a continuum with DND and are likely mediated by many of the same factors.

As in ischemic stroke, there is no accepted use of anti-inflammatory treatments for improving outcome after SAH. As discussed earlier, corticosteroids have been used to block inflammation after SAH, but there is no clear therapeutic benefit of this strategy [224]. However, there is experimental evidence from animal studies showing that blocking inflammatory pathways can improve both blood brain barrier breakdown and neuronal survival after SAH [219, 225]. In one study utilizing a rat model of SAH, there was a reduction in TNFα production, cerebral edema, and apoptosis in response to a blockade of a sulfonylurea receptor that is upregulated after SAH [45].

Clinically, the presence of cell death, cerebral edema, and vasospasm all contribute to poor outcomes after SAH. Though it is difficult to measure cell loss in humans, there is evidence for hippocampal neuronal loss after SAH based on MRI imaging, and elevated neurofilament levels in CSF correlate with functional outcome after SAH, indicating a link between axonal breakdown and clinical outcome [226, 227]. As in ischemic stroke, proinflammatory cytokines have gained attention as biomarkers for outcome in SAH, and the recent literature is well reviewed by Lad and colleagues [228]. Genetic differences in inflammatory cytokine expression and haptoglobin phenotype (discussed previously) have also been used to prognosticate outcome in patients with SAH, without definitive results [69]. Human genetic studies have also shown a benefit of lower TNFα levels for recovery after SAH [229]. This points to a possible dual role for inflammation in both acute injury and recovery, as has been proposed in other neurological diseases [230]. There is already experimental support for this dual role of inflammation in SAH, as the cytokine MCP-1 that has been associated with poor outcomes

Table 1: Key inflammatory molecules implicated in the pathology of SAH.

Molecule	Function	Roles in animal studies	Roles in human studies	Comments
Selectins	Leukocyte adhesion	Inhibition of selectins decreases vasospasm [62, 63]	Higher levels in CSF correlate to vasospasm in some studies [3], found in walls of ruptured aneurysms [36]	Variable expression in patients with SAH [3, 8, 9], used to prognosticate outcome in critically ill patients without SAH [28]
Integrins	Leukocyte adhesion	Blocking reduces vasospasm [64–66]	Higher levels seen in patients with vasospasm [3]	
TNFα	Proinflammatory cytokine produced by leukocytes	Induces neuronal apoptosis after SAH [52]; blockade reduces vasospasm [67]	Found in CSF in patients after SAH and correlates with vasospasm after SAH [68]	Variable expression in patients with SAH [2, 10–12]
MCP-1	Macrophage chemoattractant	Promotes repair of aneurysms [69]	Found in CSF after SAH and associated with poor outcomes but not vasospasm [9, 21]	Also associated with vascular injury [31]
ICAM-1	Leukocyte adhesion	Increased in animal SAH studies [47, 70]; blockade reduces vasospasm [71]	Increased in patients with SAH [3]	Used to prognosticate outcome in critically ill patients without SAH [28]
Interleukins	Mediate leukocyte interactions	Blockade reduces vasospasm [72]	Peak early in SAH [9, 11]	Peak at variable times in human studies [29, 30]
Endothelin-1	Potent vasoconstrictor	Inhibition reduces vasospasm [73, 74]	Produced by monocytes from SAH patients [13, 14], with no proved benefit in clinical trials [75]	Highly variable expression after SAH and may not correlate with vasospasm [13, 16, 76]

and vasospasm after SAH has recently been used to promote repair of experimental aneurysms [21, 231].

6. Discussion

Evidence from both clinical and animal studies indicates that inflammation contributes to aneurysm formation, brain injury, and vasospasm after SAH and that many of the same molecules contribute to vasospasm and brain injury after SAH (Figure 1, Table 1). Much of the data from human studies linking inflammation to worse outcomes after SAH is correlative and studies examining different inflammatory molecules at different time points after SAH make it difficult to make direct comparisons (Table 1). However, taken together, human and animal studies suggest that a higher "inflammatory burden" contributes to the pathophysiology of SAH. This would suggest anti-inflammatory treatment to be a robust treatment strategy for SAH, as in other diseases. For example, the possibility that aspirin could reduce chronic inflammation within the walls of aneurysms and decrease the risk of rupture [34, 35] is akin to the paradigm of human cardiovascular disease in which the anti-inflammatory actions of aspirin and statins may protect against cardiovascular disease [122, 232, 233]. Unfortunately, this strategy has not borne out reliably in clinical trials. One potential reason for this is that animal studies of homogenous populations may not be an accurate model of SAH in humans where individual responses to a given insult could be quite variable.

Clinicians who care for patients with SAH understand that there is a wide range of physiologic responses to SAH, even in patients who present with the same initial grade of hemorrhage. While this is doubtlessly influenced by many factors (such as SAH blood volume), the intensity of an individual's inflammatory response to SAH may also determine if a patient develops delayed clinical deterioration or vasospasm. While this could be influenced by factors such as haptoglobin genotype [122], there are probably other genetic and environmental factors that influence patients' production of, and tolerance to, a post-SAH inflammatory response. Evidence from animal studies has shown that inflammatory stimuli can both exacerbate and reduce vasospasm, depending on the intensity of the stimulus [234]. A recent

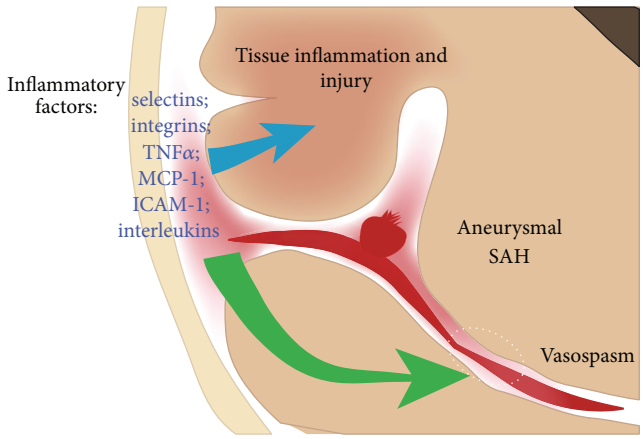

Figure 1: Schematic of a coronal projection of a ruptured cerebral aneurysm and contributing factors that result in cerebral vasospasm after SAH and delayed ischemic injury. Many inflammatory factors are hypothesized to contribute to brain injury and vasospasm after SAH. The interface between subarachnoid blood, brain parenchyma, and the cerebral vasculature is the likely location for induction of inflammatory cascades that lead to brain injury and vasospasm after SAH.

clinical study implied that preexisting atherosclerotic disease could have a protective effect on patients who suffer SAH, possibly by modifying neuroinflammation [233, 235, 236]. In the future, treatment for SAH may involve tailoring therapy to match the timing and intensity of an individual patient's inflammatory response. In order for this approach to be implemented, successful validation of inflammatory biomarkers and outcome measures for SAH would need to be developed.

7. Conclusion

The immune response within (and possibly outside of) the CNS is clearly a driving force behind many of the pathological events of SAH, including both vasospasm and early brain injury. Though much experimental and clinical work has linked increased inflammation to poor outcome after SAH, there is still no proven anti-inflammatory treatment that can be offered to patients who have suffered SAH. The volume of research on inflammation and SAH is rapidly expanding and will likely lead to new clinical trials, development of biomarkers, and hopefully anti-inflammatory treatments for SAH. Though anti-inflammatory treatments will likely improve the lives of patients with SAH, it must be remembered that neuroinflammation has beneficial effects as well and could also play a role in recovery after SAH.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Alteration of Basilar Artery Rho-Kinase and Soluble Guanylyl Cyclase Protein Expression in a Rat Model of Cerebral Vasospasm following Subarachnoid Hemorrhage

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Background and Purpose. The vasoconstrictor endothelin-1 (ET-1) has been implicated in the pathogenesis of cerebral vasospasm following subarachnoid hemorrhage (SAH). Previous results showed that CGS 26303, an endothelin converting enzyme (ECE) inhibitor, effectively prevented and reversed arterial narrowing in animal models of SAH. In the present study, we assessed the effect of CGS 26303 on neurological deficits in SAH rats. The involvement of vasoactive pathways downstream of ET-1 signaling in SAH was also investigated. *Methods*. Sprague-Dawley rats were divided into five groups (n = 6/group): (1) normal control, (2) SAH, (3) SAH+vehicle, (4) SAH+CGS 26303 (prevention), and (5) SAH+CGS 26303 (reversal). SAH was induced by injecting autologous blood into cisterna magna. CGS 26303 (10 mg/kg) was injected intravenously at 1 and 24 hr after the initiation of SAH in the prevention and reversal protocols, respectively. Behavioral changes were assessed at 48 hr after SAH. Protein expression was analyzed by Western blots. *Results*. Deficits in motor function were obvious in the SAH rats, and CGS 26303 significantly improved the rate of paraplegia. Expressions of rho-kinase-II and membrane-bound protein kinase C-δ and rhoA were significantly increased, while those of soluble guanylyl cyclase α_1 and β_1 as well as protein kinase G were significantly decreased in the basilar artery of SAH rats. Treatment with CGS 26303 nearly normalized these effects. *Conclusions*. These results demonstrate that the rhoA/rho-kinase and sGC/cGMP/PKG pathways play pivotal roles in cerebral vasospasm after SAH. It also shows that ECE inhibition is an effective strategy for the treatment of this disease.

1. Introduction

Subarachnoid hemorrhage (SAH) is an important subcategory of stroke due to an unacceptably high mortality rate as well as the severe complications it causes, such as cerebral vasospasm, neurological deficit, and cardiopulmonary abnormality [1]. The potent vasoconstrictor endothein-1 (ET-1) has been implicated in the pathogenesis of this disease [2]. One strategy to inhibit the biological effect of ET-1 is by means of its receptor antagonists. In fact, various selective and nonselective ET-1 receptor antagonists have been evaluated in animal models of cerebral vasospasm following SAH and in humans with varying degrees of success [3, 4]. An alternative approach to ameliorate the deleterious effects of ET-1 is to suppress the production of this vasoconstrictor by inhibiting endothelin-converting enzyme (ECE), which catalyzes the final step of ET-1 biosynthesis. CGS 26303 is such an inhibitor [5–8], and it has been shown to prevent and reverse cerebral vasospasm in a rabbit model of SAH [9].

The involvement of other vasoactive pathways downstream of ET-1 signaling in SAH is not completely understood. It has been shown that ET-1 potentiates the contraction of cerebrovascular smooth muscles induced by oxyhemoglobin, a blood clot component and major causative factor in cerebral vasospasm, via the protein kinase C (PKC) and rhoA/rho kinase pathways [11]. This is consistent with the finding that the PKC inhibitor staurosporine abolished ET-1-induced contraction in rabbit basilar artery [12]. In addition, lines of evidence accumulated to date have suggested that activation of protein kinase C (PKC) plays a role in the delayed and prolonged contraction of major arteries after SAH [13–15]. For example, phorbol 12,13-diacetate, a PKC activator, induced a potent and long-lasting contraction of the canine basilar artery [16]. In a two-hemorrhage canine model of cerebral vasospasm, translocation of PKCδ from the cytosol to membrane in the basilar artery was noted after the second injection of autologous blood on day 4 where severe vasospasm occurred, suggesting that this isoform of PKC was activated [17]. Furthermore, injection of the PKCδ inhibitor rottlerin into the cisterna magna on day 4 before the second hemorrhage inhibited this vasospastic response and PKCδ translocation [18].

In contrast, the effect of elevated ET-1 in SAH on the rho-kinase pathway has attracted less attention despite the documented involvement of rho-kinase in cerebral vasospasm following SAH [19, 20]. Rho is a family of small G-proteins consisting of 3 members, that is, rhoA, rhoB, and rhoC, that play a substantial role in intracellular signaling [10, 21]. Under unstimulated conditions, rho is in an inactive GDP-bound form and resides mainly in the cytosol. In vascular smooth muscle cells stimulated by vasoactive agents, rho undergoes GDP-GTP exchange to become activated with a subsequent translocation to the cell membrane where it interacts with its downstream effectors such as rho-kinase (ROCK). There are two isoforms of rho-kinase, namely, ROCK-I and ROCK-II. Activation of rho-kinase promotes smooth muscle contraction by phosphorylation of myosin light chain phosphatase (MLCP) at the myosin-binding subunit, resulting in inhibition of the phosphatase activity [10, 21]. In a canine two-hemorrhage model of cerebral vasospasm, topical application of a specific inhibitor of rho-kinase Y-27632 dose-dependently decreased the spastic response, rho-kinase activity, and phosphorylation of MLCP in the basilar artery [22].

Besides ET-1, the vasodilator nitric oxide (NO) produced by nitric oxide synthase in endothelium is also an important regulator of the cerebral vascular tone [23]. Upon synthesis, NO activates soluble guanylyl cyclase (sGC), a heterodimeric enzyme consisting of α (α_1, α_2, and α_3) and β (β_1, β_2, and β_3) subunits [24, 25]. Activation of sGC leads to the production of cGMP, which in turn activates cGMP-dependent protein kinase (PKG) among other targets and ultimately results in smooth muscle relaxation. Under physiological conditions, normal production of ET-1 and NO yields a balanced cerebral vascular tone. However, enhanced generation of ET-1 along with impairment in NO production or in the vasodilatory response to NO was noted in humans and animals with SAH [13]. Nevertheless, the effect of inhibition of ET-1 production on the sGC/cGMP pathway has not been fully investigated.

In the present study, we aimed to assess the neurological deficits, plasma ET-1 levels, and the expressions of PKCδ, rhoA, ROCK-II, and sGC/cGMP/PKG in the basilar artery of rats subjected to experimental SAH. The effect of CGS 26303 on the neurological deficits and vasoactive pathways downstream of ET-1 signaling in SAH was also investigated.

2. Materials and Methods

2.1. Materials. Anti-mouse β-actin antibodies and anti-rabbit sGCα_1 and sGCβ_1 antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-rabbit PKG, anti-mouse PKCδ, anti-rabbit ROCK-II, anti-mouse rhoA, and horseradish peroxidase-labeled goat anti-mouse IgG antibodies were purchased from Abcam (Cambridge, MA, USA), BD Transduction Lab (San Jose, CA, USA), Upstate Biotech (Lake Placid, NY, USA), Santa Cruz Biotech (Santa Cruz, CA, USA), and Chemicon International (Temecula, CA, USA), respectively. CNM protein extraction kits were products of Biochain (Hayward, CA, USA). ET-1 and cGMP ELISA kits were obtained from Assay Designs (Farmingdale, NY, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. CGS 26303 was provided by Dr. Arco Y. Jeng (Novartis Pharmaceuticals, East Hanover, NJ, USA).

2.2. Animal Protocols. All animal procedures were approved by the Kaohsiung Medical University Hospital animal research committee. Thirty male Sprague-Dawley rats (Bio-Lasco, Taiwan) weighing 250–300 g were divided into the following five groups (n = 6/group): Group 1, control animals (PBS); Group 2, rats subjected to SAH; Group 3, SAH rats treated with vehicle (0.1 mol/L NaOH/PBS); and Groups 4 and 5, SAH rats treated with CGS 26303 (10 mg/kg, i.v.) at 1 hr (prevention protocol) and 24 hr (reversal protocol) after SAH, respectively. To induce SAH, rats were anesthetized with a mixture of KetaVed (55 mg/kg) and xylazine (9 mg/kg) intraperitoneally (i.p.), and fresh blood (1 mL/kg) was drawn from the central tail artery and injected into the cistern magna according to a published protocol [8]. The mortality rate after induction of SAH was 15–20%, and it was the same in all of the SAH groups.

2.3. Hemodynamic Measurements. Heart rate and blood pressure were monitored before and after CGS 26303 treatment as well as at 48 hr after the induction of SAH by a tail-cuff method.

2.4. Neurological Assessment. Neurological assessment was performed before and at 48 hr after the induction of SAH. Motor function was quantified by assessment of ambulation and placing and stepping responses using a scoring system published previously and shown in Table 1(a) [26].

2.5. Determination of Plasma ET-1 and Tissue cGMP Levels. Blood was collected in heparin-containing tubes prior to sacrifice. Plasma samples were frozen at −70°C until use. ET-1 was determined using an ELISA kit according to the

Table 1: Behavioral changes induced by experimental subarachnoid hemorrhage in the rat.

(a) Scoring system used for motor function assessment

Motor function	Behavior	Score
Ambulation	Normal (symmetric and coordinated)	0
	Toes flat under the body while walking with ataxia	1
	Knuckle-walking	2
	Movement in lower extremities but unable to knuckle walk	3
	No movement, dragging lower extremities	4
Placing/stepping reflex	Normal (coordinated lifting and placing response)	0
	Weak response	1
	No stepping	2

Ambulation was assessed by walking with lower extremities, while placing/stepping reflex was evaluated by dragging the dorsum of the hind paw over the edge of a surface [10].

(b) Effect of CGS 26303 on behavioral changes induced by experimental subarachnoid hemorrhage in the rat

Group	Ambulation	Placing/stepping reflex	Motor deficit index (MDI)	Paraplegia rate
Normal (no SAH)	0*	0*	0*	0*
SAH	1.27 ± 0.18	1.50 ± 0.13	2.36 ± 0.20	67
SAH + vehicle	1.20 ± 0.13	1.47 ± 0.13	2.27 ± 0.18	58
SAH + CGS 26303 (P)	0.75 ± 0.13*	0.71 ± 0.17*	1.33 ± 0.18*	30*
SAH + CGS 26303 (R)	0.85 ± 0.15*	0.83 ± 0.16*	1.40 ± 0.15*	23*

Subarachnoid hemorrhage (SAH) was induced in rats by injecting autologous blood into the cisterna magna. CGS 26303 was administered intravenously at a dose of 10 mg/kg at 1 (prevention protocol, P) or 24 (reversal protocol, R) hr after SAH. The motor function was assessed using the scoring system shown in Table 1(a) and was performed at 48 hr after SAH prior to sacrifice. Six animals were used in each group, and motor function assessment was performed five times for each animal. MDI is the sum of scores from ambulation and placing/stepping reflex. The paraplegia rate is defined as the percentage of rats with MDI ≥ 3 in each group ($n = 6$). *$P < 0.05$ versus the SAH group.

instruction of the manufacturer. cGMP in the homogenate of basilar artery was measured by an ELISA kit.

2.6. Tissue Morphometry. At 48 hr after the induction of SAH, the animals were anesthetized by chloral hydrate (0.3 mg/kg, i.p.). Perfusion-fixation was performed according to a published protocol [9]. Basilar arteries were harvested from the brainstems, and the middle third of each artery was dissected for morphometric analysis. The rest of tissue was frozen in liquid N_2 and stored at −70°C until use for measurements of protein expression and cGMP levels.

2.7. Protein Expression. Basilar arteries were homogenized in buffers C, N, and M for extraction of cytoplasmic, nuclear, and membrane-bound proteins, respectively, according to the instructions of the manufacturer. Expressions of PKCδ, rhoA, ROCK-II, sGCα_1, sGCβ_1, and PKG were determined by Western blots using specific antibodies according to the instructions of the respective manufacturers.

2.8. Statistical Analyses. Group data are expressed as the means ± SEM. Comparison of the neurological deficit scores between groups was performed by the Mann-Whitney test. Comparison of protein expression and biomarkers between groups was done using one-way ANOVA followed by Dunnett's test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. General Observations. No statistically significant differences in the body weight, heart rate, or blood pressure were found among the 5 groups at the end of the experiments (results not shown). Visual inspection during the removal of the brain showed that subarachnoid clots had formed and covered the basilar artery in all animals subjected to SAH.

3.2. Neurological Deficit. Using the scoring system shown in Table 1(a), both the ambulation and placing/stepping reflex scores in the SAH and SAH+vehicle groups were significantly higher than in the controls (Table 1(b)). The sum of scores from these two tests is referred to as motor deficit index (MDI). The values of MDI in the SAH and SAH+vehicle groups were 2.36±0.20 and 2.27±0.18, respectively, compared with a score of 0 in the normal control. Treatment with CGS 26303 significantly improved the MDI in the prevention and reversal groups (Table 1(b)). Likewise, paraplegia rate (defined as the percentage of rats with MDI ≥ 3 in each group) was substantially decreased in both the CGS 26303 treatment groups when compared with the SAH animals (Table 1(b)).

3.3. Plasma ET-1 Levels. When compared with controls, plasma ET-1 levels in the SAH group were significantly elevated (Figure 1(a)). Injection with CGS 26303 drastically decreased plasma ET-1 in both the prevention and reversal groups to levels that were not statistically different from the control.

3.4. Tissue Morphometry. The internal elastic lamina in the basilar artery of SAH and SAH+vehicle groups showed substantial corrugation when compared with that obtained from controls (Figure 1(b)). Corrugation was significantly

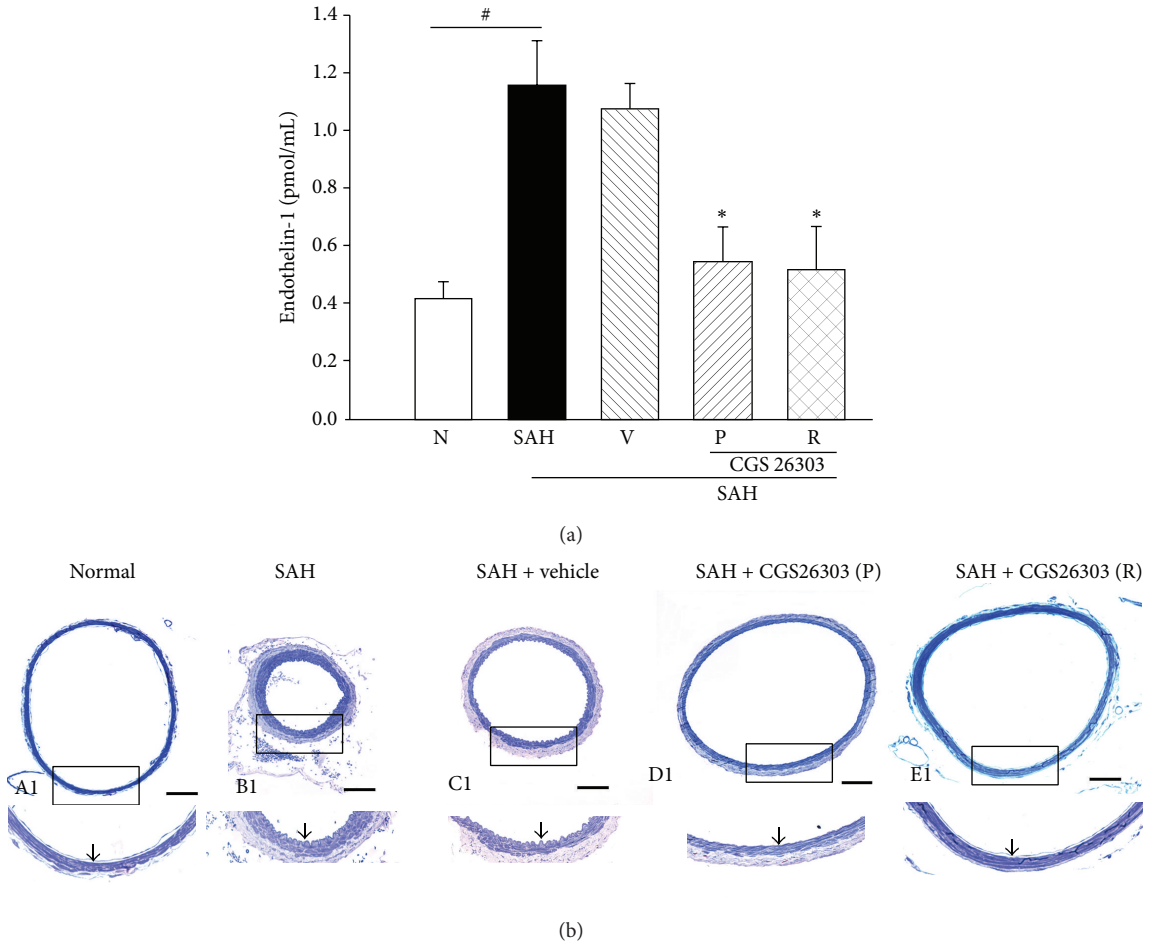

Figure 1: Effect of CGS 26303 on plasma ET-1 levels (upper panel) and cross section of basilar artery (lower panel). CGS 26303 was administered at 1 (prevention protocol, P) or 24 (reversal protocol, R) hr after the induction of SAH. Plasma levels of ET-1 were measured by ELISA. Data are mean ± SEM ($n = 6$/group). $^{\#,*}P < 0.05$ versus SAH. N: normal control; V: vehicle group. No statistically significant difference was found between the SAH and vehicle groups. The cross sections in the SAH+CGS 26303 group were obtained from animals that underwent the prevention protocol. The arrowheads show the endothelial layer and basal lamina. Scale bars in the first and second rows of the micrograms represent 5 and 0.25 μm, respectively.

less prominent in the SAH+CGS 26303 groups. The cross-sectional areas of basilar artery in the SAH and SAH+vehicle groups were significantly reduced when compared with the control group. Treatment with CGS 26303 significantly attenuated the decrease in both the prevention and reversal groups (results not shown).

3.5. Translocation of PKCδ. It has been shown that PKCδ translocates from the cytosolic compartment to become membrane-bound upon activation. The ratio of PKCδ membrane to cytosolic expression in the basilar artery of the SAH rats was set at 100% as a reference. Only about 60% of PKCδ was membrane-bound in normal animals, treated with CGS 26303 either at 1 or 24 hr after SAH inhibited the translocation of PKCδ to levels similar to that of the normal control (Figure 2).

3.6. RhoA Translocation and ROCK-II Expression in the Basilar Artery. Similar to that seen with PKCδ, rhoA also translocates from the cytosolic compartment to become membrane-bound upon activation. In the basilar artery, membrane-bound rhoA was significantly greater in rats subjected to SAH when compared with the normal control (Figure 3(a)). Treatment with vehicle had no effect, while CGS 26303 significantly reduced membrane-bound rhoA.

The pattern of ROCK-II expression in the basilar artery resembled that observed with levels of membrane-bound PKCδ or rhoA. ROCK-II expression was significantly increased in the SAH and SAH+vehicle groups when compared with controls, and treatment with CGS 26303 normalized the expression of ROCK-II (Figure 3(b)).

3.7. Expression of sGC and Its Downstream cGMP/PKG Pathway in the Basilar Artery. In contrast to an increased expression of ROCK-II in the basilar artery of rats subjected to SAH, the expressions of sGCα_1 and sGCβ_1 were significantly decreased when compared with the controls (Figure 4). Treatment with CGS 26303 normalized the expressions of

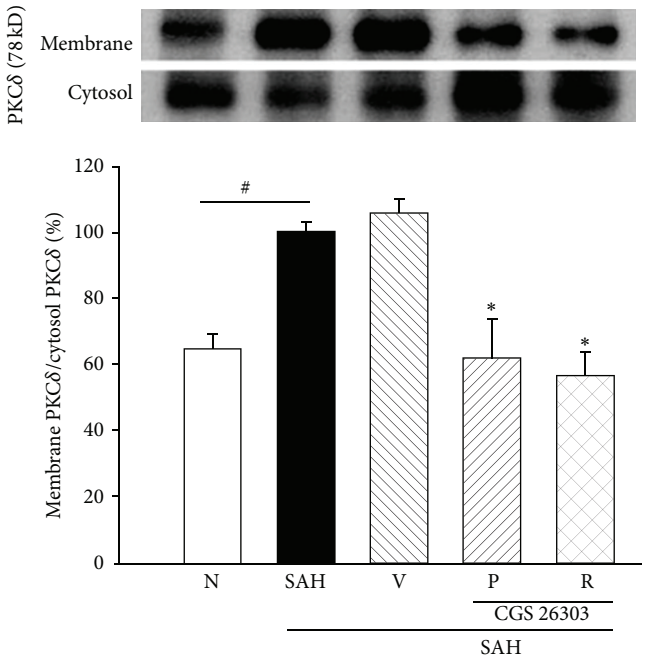

Figure 2: Inhibition of PKCδ translocation by CGS 26303 in the basilar artery. Expression of PKCδ in the cytosolic and membrane compartments of basilar artery was determined by Western blot analysis. The ratio of membrane-bound to cytosolic PKCδ in the SAH group was set at 100%. Data are mean ± SEM (n = 6/group). $^{\#,*}P < 0.05$ versus SAH. No statistically significant difference was found between the SAH and vehicle groups. All groups are identical to those shown in the legend of Figure 1.

these two enzymes to levels that were not statistically different from the controls (Figure 4).

Consistent with reduced expressions of sGCα_1 and sGCβ_1 in the basilar artery of SAH rats, the levels of cGMP were also significantly decreased in these animals (Figure 5(a)). Administration of CGS 26303 in both the prevention and reversal protocols significantly attenuated the reduction of cGMP production in the basilar artery of the SAH animals (Figure 5(a)). This increased production of cGMP upon CGS 26303 treatment also resulted in an increased expression of PKG in the basilar artery of the SAH rats which, if untreated, showed a significant reduction in PKG expression when compared with the controls (Figure 5(b)).

3.8. Expressions of ROCK-II, sGCα_1, and sGCβ_1 in the Brain, Heart, and Lung. It has been shown that ROCK and sGC have a wide distribution in the brain, especially in the cortex, hippocampus, and cerebellum [27, 28]. The expressions of ROCK-II, sGCα_1, and sGCβ_1 in these regions as well as in the brain stem were investigated. Furthermore, the expressions of these enzymes were examined in the heart and lung, since an increase in the levels of plasma ET-1 was found in the SAH rats and ET-1 was shown to play pathogenic roles in various cardiac and pulmonary diseases.

Unexpectedly, no significant changes in the expressions of ROCK-II, sGCα_1, and sGCβ_1 were found in these tissues examined in the SAH rats when compared with the control animals, except for an increased expression of ROCK-II in the heart (results not shown). Interestingly, no effects on the expressions of these enzymes were detected upon treatment with CGS 26303 in the SAH animals.

4. Discussion

Cerebral vasospasm following SAH is the leading cause of death and disability after aneurysm rupture. Despite the extensive research and numerous clinical studies conducted, the neurological outcomes in various trials for patients suffering from SAH remain disappointing [29]. Furthermore, assessment of neurological deficits in preclinical studies is scarce. In this regard, the results reported in the present study showing that an ECE inhibitor CGS 26303 significantly improved the motor function index and the rate of paraplegia in the SAH rats are significant findings. In addition, treatment with CGS 26303 decreased the activation of PKCδ and rhoA as well as the expression of rho-kinase, factors thought to contribute to the spastic response, while it concomitantly enhanced components in the sGC/cGMP/PKG pathway. These results suggest that the PKC, rhoA/rho-kinase, and sGC/cGMP/PKG pathways may play important roles in cerebral vasospasm after SAH and that the beneficial effects of CGS 26303 in cerebral vasospasm following SAH might be due to additive influence on all three pathways.

As described herein, the levels of plasma ET-1 were significantly increased with concomitant activation of the PKCδ and rhoA/rho kinase-II pathways in the basilar artery of rats subjected to SAH. Treatment with the ECE inhibitor CGS 26303 normalized plasma ET-1 as well as the expression of the two vasoconstrictive pathways. These results are consistent with the reports showing that oxyhemoglobin is a major causative component of blood clot for cerebral vasospasm following SAH [30, 31] and that ET-1 potentiates the oxyhemoglobin-induced cerebrovascular smooth muscle contraction via the rhoA/rho kinase and PKC pathways [11]. In the present study, the expression of rho kinase-II in the heart, but not in various regions of the brain and the lung, was also activated upon induction of SAH. Interestingly, the expression of ROCK-II in the heart appears to be ET-1-independent as treatment with CGS 26303 had no effect.

In contrast to the activation of the two aforementioned vasoconstrictive pathways, SAH resulted in decreased expression of the vasodilatory pathway sGC/cGMP/PKG (Figures 4 and 5). This pathway has been documented as NO-mediated. However, in a previous study, neither neuronal nor endothelial NO synthase mRNA expression in the brain of the SAH rats was significantly different from that in the brain of the controls and treatment with CGS 26303 had no effect [32]. Nevertheless, it is worth noting that, in addition to its ECE inhibitory activity, CGS 26303 also inhibits the activity of neutral endopeptidase (NEP) [17, 33], which degrades potent vasodilators such as atrial natriuretic peptide (ANP) [34]. In the present study it is possible that an elevated ANP level due to CGS 26303 treatment stimulates the sGC/cGMP/PKG pathway via increased production of NO through the inducible NO synthase as seen in the neonatal rat cardiac myocytes [35].

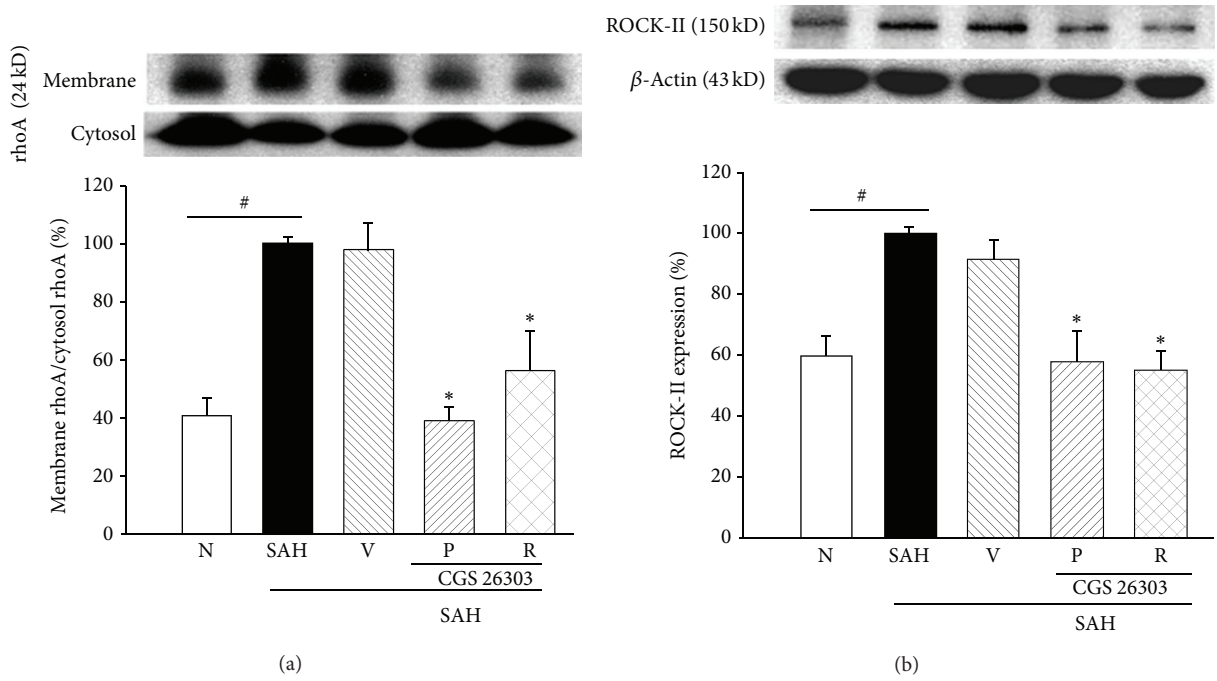

Figure 3: Inhibitory effect of CGS 26303 on rhoA translocation (a) and ROCK-II expression (b) in the basilar artery. Expression of rhoA in the cytosolic and membrane compartments as well as ROCK-II was determined by Western blot analysis. The ratio of membrane-bound to cytosolic rhoA in the SAH group was set at 100% in (a), whereas the expression of ROCK-II (normalized using β-actin) in the same group was set at 100% in (b). Data are mean ± SEM ($n = 6$/group). $^{\#,*}P < 0.05$ versus SAH. No statistically significant difference was found between the SAH and vehicle groups. All groups are identical to those shown in the legend of Figure 1.

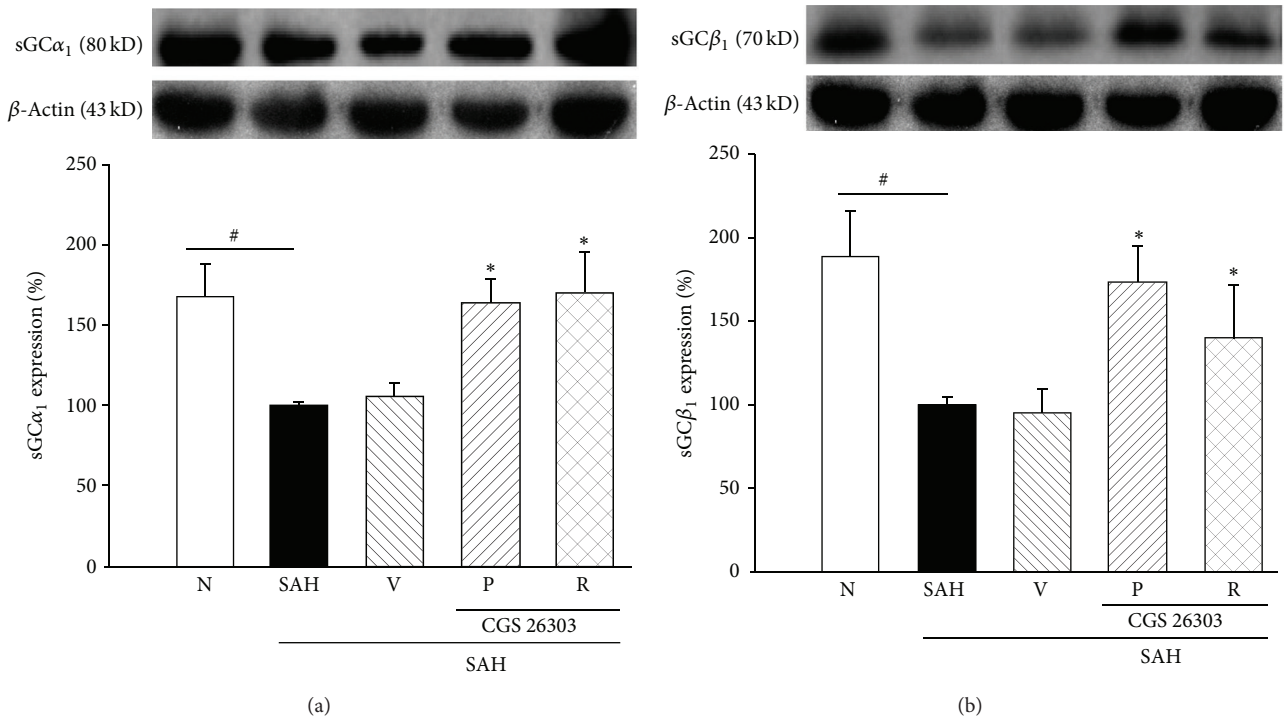

Figure 4: Upregulation of sGCα_1(a) and sGCβ_1 (b) by CGS 26303 in the basilar artery. Expression of sGCα_1 and sGCβ_1 was determined by Western blot analysis and normalized using β-actin. sGCα_1 and sGCβ_1 expressions in the SAH group were set at 100%. Data are mean ± SEM ($n = 6$/group). $^{\#,*}P < 0.05$ versus SAH. No statistically significant difference was found between the SAH and vehicle groups. All groups are identical to those shown in the legend of Figure 1.

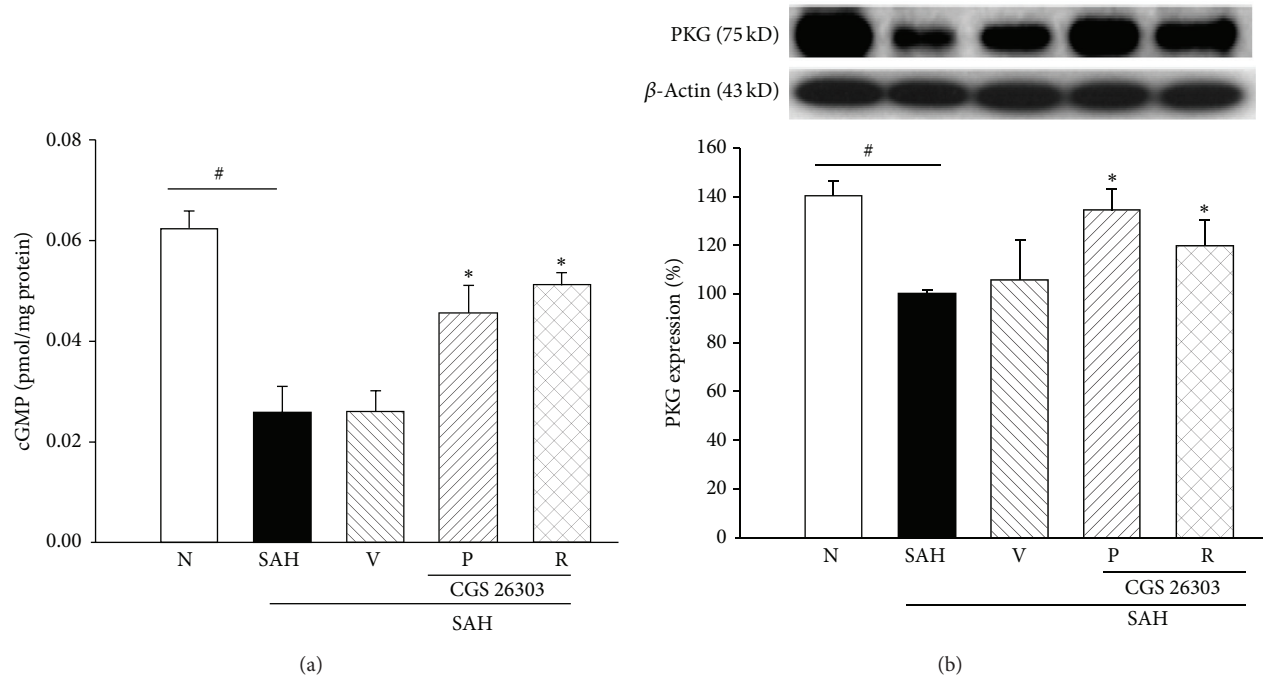

Figure 5: Increased levels of cGMP (a) and PKG expression (b) by CGS 26303 in the basilar artery. The levels of cGMP were measured by ELISA. Expression of PKG was determined by Western blot analysis. PKG expression in the SAH group was set at 100%. Data are mean ± SEM ($n = 6$/group). $^{\#,*}P < 0.05$ versus SAH. No statistically significant difference was found between the SAH and vehicle groups. All groups are identical to those shown in the legend of Figure 1.

5. Conclusion

In summary, this study shows that CGS 26303 reduced the levels of plasma ET-1, improved the motor function index, decreased the activation of the vasoconstrictive PKCδ and rhoA/rho-kinase pathways, and activated the vasodilatory sGC/cGMP/PKG pathway in rats subjected to SAH. It is likely that this compound exerts these beneficial effects via its dual ECE/NEP inhibitory activities. However, confirmation of the utility of CGS 26303 for the treatment of cerebral vasospasm following SAH awaits future clinical studies.

Conflict of Interests

The authors have no conflict of interests or financial disclosures. No part of this paper has been published/presented elsewhere.

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Review Article

To Look Beyond Vasospasm in Aneurysmal Subarachnoid Haemorrhage

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Delayed cerebral vasospasm has classically been considered the most important and treatable cause of mortality and morbidity in patients with aneurysmal subarachnoid hemorrhage (aSAH). Secondary ischemia (or delayed ischemic neurological deficit, DIND) has been shown to be the leading determinant of poor clinical outcome in patients with aSAH surviving the early phase and cerebral vasospasm has been attributed to being primarily responsible. Recently, various clinical trials aimed at treating vasospasm have produced disappointing results. DIND seems to have a multifactorial etiology and vasospasm may simply represent one contributing factor and not the major determinant. Increasing evidence shows that a series of early secondary cerebral insults may occur following aneurysm rupture (the so-called *early brain injury*). This further aggravates the initial insult and actually determines the functional outcome. A better understanding of these mechanisms and their prevention in the very early phase is needed to improve the prognosis. The aim of this review is to summarize the existing literature on this topic and so to illustrate how the presence of cerebral vasospasm may not necessarily be a prerequisite for DIND development. The various factors determining DIND that worsen functional outcome and prognosis are then discussed.

1. Introduction

SAH accounts for only 5% of all strokes, with an incidence of nine per 100,000 person years [1]. Half the patients are younger than 55 years and therefore SAH has a severe economic and social impact [2]. One in six patients die during the sudden onset of bleeding and those who survive may die or deteriorate owing to early aneurysmal rebleeding, secondary delayed cerebral ischemia (DCI), hydrocephalus, or medical complications. Approximately 70% of patients die or subsequently need help with the ordinary activities of daily life [3].

Secondary DCI occurs in c. 30% of all patients [4] and results in poor outcome in half of these [3]. The high risk period for DIND is between 4 and 10 days after aneurysm rupture [4]; the pathogenesis is still incompletely understood, but classically it has been attributed to cerebral vasospasm [5].

During the last century there was a wide consensus that cerebral vasospasm was the most important determinant of poor prognosis in patients with aSAH [6]. Research was mainly directed to control and prevent delayed vasospasm, often with disappointing results. Indeed radiological improvement of vasospasm does not correlate clearly with changes in functional outcome [7, 8]. Over the last decade, growing experimental and clinical evidence has demonstrated that the presence of delayed vasospasm of the major cerebral vessels may indeed be a contributing factor but not necessarily the principal determinant of DCI and DIND. Indeed, cerebral infarction can also occur when vasospasm is not angiographically detected in the territorial artery [9] and poor outcome in aSAH seems to be directly dependent

on infarction but independent of vasospasm [10]. There is increasing evidence that other coexisting factors may be involved in the development of DIND and their characterisation and treatment could improve the consistently poor clinical outcome in patients with aSAH.

The aim of this review is to discuss the various mechanisms contributing to the poor prognosis in patients with SAH and to redefine the role of delayed cerebral vasospasm in DIND.

2. Early Brain Injury

The term early brain injury (EBI) was first coined in 2004 to explain the acute pathophysiological events occurring within 72 hours of aSAH that begin minutes after bleeding commences [11, 12]. These events include cerebral autoregulation and blood-brain barrier (BBB) disruption, activation of inflammatory pathways, excitotoxicity, oxidative stress, and activation of apoptosis [13]. These are direct effects of the presence of blood in the subarachnoid space and also of transient cerebral ischemia. Brain injury is not limited to the primary site of haemorrhage; many of the mechanisms occurring with EBI contribute to the pathogenesis of delayed ischemic injury and are thus responsible for subsequent poor outcome. Hence early detection of EBI may make it possible to predict patient outcome; logically, therefore, early intervention that inhibits such changes may decrease mortality and improve overall outcome.

2.1. Mechanical Injury and Cerebral Autoregulation Disruption. Immediately after an aSAH a reactive constriction of the artery supplying the ruptured aneurysm occurs, thus producing a mechanical injury [14]. The consequence is an acute global ischemia leading to BBB disruption through endothelial cell death [15, 16]. Furthermore, both vasogenic and cytotoxic brain oedema may contribute by elevating intracranial pressure (ICP) and impairing cerebral blood flow (CBF).

Early elevation of ICP values is common after SAH. Two patterns of ICP elevation, namely, transitory and sustained, are described. The extent of rise in ICP is often used to predict outcome in SAH [17] and sustained ICP elevation is associated with higher mortality [18]. This phenomenon is associated with a severe reduction in CBF, cerebral perfusion pressure (CPP), and impaired cerebral autoregulation [19, 20], thus increasing mortality rates. Bederson et al. observed that CBF reduction to less than 40% of baseline in the first hour after SAH predicted 100% mortality, independent of ICP and CPP values [11].

2.2. Electrolyte Disturbances. Electrolyte disturbances are often observed within the first hours after SAH and they may be responsible for several mechanisms of EBI. Hyponatremia develops within 1-2 days from the initial bleed [21] and it occurs in 10%–30% of patients at admission; it is caused by a cerebral salt-wasting syndrome and inappropriate secretion of antidiuretic hormone. The treatment of hyponatremia is not easy and clinical signs may mimic DIND. Furthermore hyponatremic patients have a risk of developing delayed ischemic injury three times higher than normonatremic patients [22]. Risk factors for hyponatremia include a history of diabetes, chronic heart, hepatic failure, adrenal insufficiency, and NSAIDs or diuretic use [23].

Cellular calcium homeostasis is impaired in neuronal, cerebral endothelial, and smooth muscle cells; the intracellular elevation is due to *N-methyl-D-aspartate* (NMDA) glutamate receptor activation and deregulation of adenosine triphosphatase (ATPase) dependent channels. Pathological rise in intracellular calcium may result in persistent contraction of smooth muscle cells in cerebral arteries, also causing glutamate release and activation of apoptotic pathways [24] (Figure 1).

Approximately 40% of patients admitted within 48 hours after SAH have abnormally low serum magnesium [25]. Magnesium decrease contributes to the rise in intracellular calcium by blocking NMDA receptors in an activated state and this provokes vasoconstriction, platelet aggregation, release of excitatory aminoacids, and increased synthesis of endothelin-1 (ET-1) [26].

A high level of serum potassium has been detected after SAH [27], probably owing to decreased activity in the potassium-sodium pump mechanism. Subarachnoid haemoglobin combined with a high concentration of potassium may cause widespread constriction of cerebral arteries and a pathological decrease in CBF.

2.3. Excitotoxicity. The increased interstitial glutamate concentration after SAH is linked to cellular leakage, altered synaptic transmission, BBB disruption, and decreased glutamate uptake [28]. In animal experiments an excitotoxicity from excessive activation of ionotropic and metabotropic glutamate NMDA receptors [29] was observed, leading to excessive intracellular calcium influx and activation of apoptotic pathways [30]. The NMDA receptor-antagonist, felbamate, improved neurological performance in rat models, limiting BBB disruption [31] and development of delayed vasospasm [32]. Similarly blood glutamate scavengers have been shown to improve neurological outcome in animal models, but the blockade of NMDA receptors may actually hinder neuronal survival [33]. In clinical studies glutamate elevation in cerebral interstitial fluid detected with microdialysis was predictive of ischemia [34] and the release of excitatory amino acid after SAH measured in interstitial and cerebrospinal fluid (CSF) correlated strongly with ICP elevation, secondary brain injury, and poor outcome [35].

2.4. Nitric Oxide Alterations and Endothelin-1 Increase. Alterations in nitric oxide (NO) pathways are described in the early period after aSAH both in animals and humans. [36, 37] NO is produced by nitric oxide synthase (NOS) which can be distinguished between endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS). NO plays an important role in regulating vascular hemodynamic activity; it dilates vessels by blocking intracellular calcium release from the sarcoplasmic reticulum in smooth muscle cells and it inhibits platelet aggregation and leucocyte adhesion to the endothelial

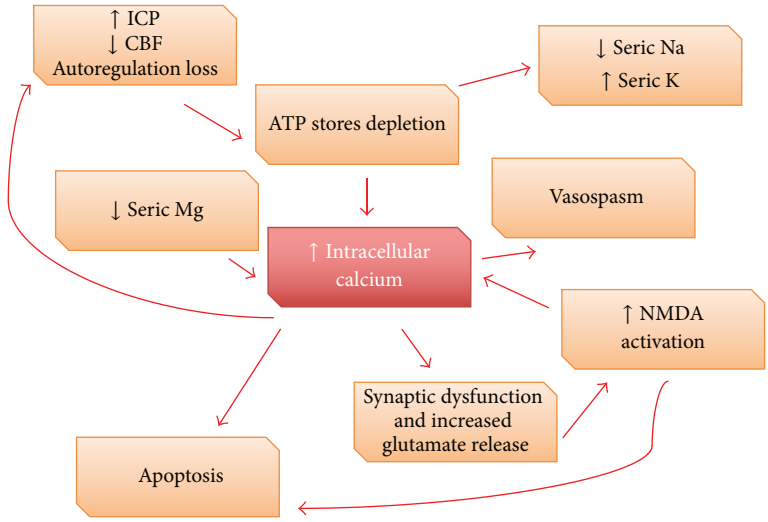

Figure 1

layer. Its alteration may disrupt autoregulation homeostasis and may be related to the pathogenesis of delayed vasospasm [37]. Animal studies demonstrate that cerebral NO level decreases within 10 min of aSAH [36] and it increases excessively after 24 hours [38]. The decreased availability of NO may be attributed to nNOS destruction and inhibition of eNOS through the presence of subarachnoid haemoglobin. A downregulation of eNOS and loss of nNOS in spastic arteries after SAH have indeed been demonstrated [39].

In clinical studies, increased cerebral NO levels are found 24 hours after aSAH and this indicates a poor prognosis [37, 40]. Inflammation activates iNOS and NO production may act as a vasodilator, in the form of peroxynitrite or as free radical itself, causing an oxidative stress in the vascular wall at the critical moment [41].

Endothelin-1 (ET-1) is the most potent endogenous activator of vasoconstriction, through the activation of calcium-dependent and independent mechanisms. The level of ET-1 increases in serum and plasma within minutes after SAH with a peak 3-4 days after injury [42]; it is physiologically produced by the endothelium, but in SAH there is an excessive release by astrocytes during the period of initial ischemia [43]. An upregulation of its receptors is equally observed in the delayed phase; ET_A receptor in particular is expressed predominantly on smooth muscle cells and is crucial in vasoconstriction and cell proliferation. ET-1 can produce long lasting vasoconstriction directly [44] and can induce morphological changes such as fibrosis or hyperplasia in the vascular wall [45]. Furthermore, a disequilibrium between NO and ET-1 level leads to unopposed vasoconstriction and promotes vasospasm development [46].

2.5. Oxidative Stress. Reactive oxygen species (ROS), principally oxygen free radicals, and reactive nitrogen species (RNS) are both linked to a number of vascular disease states. Oxidative stress plays a significant role in EBI. Animal and human studies indicate that ROS are generated early after SAH resulting in haemoglobin autooxidation and lipid peroxidation and a consequent rapid consumption of enzymatic and nonenzymatic antioxidant defence systems [47]. Such oxidative stress may be the trigger for a number of deleterious pathophysiological changes including structural alterations in endothelial cells, endothelial dysfunction and proliferation of smooth muscle cells [48], disruption of BBB, activation of the inflammatory cascade, and production of powerful local vasoconstrictors (e.g., leukotriene C4 and prostaglandin D2). [47]. The vasodilator effect of bradykinin in cerebral vessels through an inhibition by ROS further supports such a hypothesis [49]. The treatment of oxidative stress during the short effective therapeutic window that exists is difficult; injury caused by free radicals may well occur before a patient can receive effective treatment [50].

2.6. Inflammatory Pathways. The correlation between inflammation and the presence of blood in the subarachnoid space was established over fifty years ago. In 1955 Walton demonstrated that febrile patients with SAH have a worse final outcome than afebrile patients [51]. SAH triggers an inflammatory cascade: a systemic leucocytosis is commonly observed [52] and white cells can directly promote free radical formation, release cytokines and chemotactic factors to propagate the immunological response, and produce ET-1 and leukotrienes [53] and consume NO. Furthermore Spallone et al. have shown how leucocyte concentrations are more elevated in the CSF of patients with SAH-related ischemia when compared to controls [52]. By analogy elevated serum C-reactive protein levels on admission are known to be related to poor prognosis and the occurrence of delayed vasospasm [54].

Subarachnoid blood is a stimulant for nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), which mediates the transcription of multiple components of the inflammatory cascade, including adhesion molecules, cytokines, and complement [55].

Tumor necrosis factor-alpha (TNFα) may also have a critical role in determining EBI. According to Starke et al., TNFα contributes to the formation and rupture of the aneurysm and inhibitors of TNFα may therefore be beneficial not only in preventing aneurysmal progression and rupture [56], but also in limiting the inflammatory process after subarachnoid bleeding.

Cytokines and chemokines may be implicated in the development and maintenance of neurovascular injury with an early increase at six hours and a late peak between 48 and 72 hours. Their elevation in serum, CSF, and microdialysis fluid is related to early and delayed ischemia and poor outcome [44, 57].

In particular, as shown by Muroi et al., higher serum interleukin-6 (IL-6) levels are associated with worse clinical outcome and DIND. Thus it is feasible that IL-6 levels may also be used as a marker to monitor clinical progression [58].

The serum and CSF levels of endothelial adhesion molecules (in particular E-selectin, ICAM, and VCAM-1), which are vital to the capture, rolling, transmigration, and diapedesis of leucocytes to the site of inflammation, are significantly elevated after aSAH [59]. Their increase within the first three days of haemorrhage is associated with poor outcome [60].

A quantitative correlation between the degree of inflammatory response and the prognosis in patients with SAH may therefore be possible.

2.7. Blood Breakdown Products. Haemoglobin may cause vasoconstriction by direct oxidative stress (as oxy-Hb or as bilirubin oxidation products (BOXes)) [61] and also by altering the balance between NO and ET-1. Oxy-Hb is a strong spasmogenic substance; it causes prolonged contraction of smooth muscle cells when applied to cerebral arteries in vivo and antagonists seem to prevent the occurrence of vasospasm [62]. It can catalyse the generation of superoxide and hydrogen peroxide, resulting in subsequent lipid peroxidation. Furthermore haemoglobin may scavenge nitric oxide, destroy nNOS, and alter eNOS functionality and it may indeed stimulate ET-1 production [63].

Bilirubin formation is maximal during the third or fourth day after SAH and BOXes reach a maximal concentration during the major vasospasm period (4–11 days). However, BOXes seem to be potentiators rather than initiators of vasospasm [64].

The role of iron in early brain injury after SAH was investigated by Lee et al. [65]; they showed how iron chelator desferroxamine halved mortality, attenuated DNA damage, and lessened induction of iron-handling proteins in experimental models [66]. Treatment was efficacious as early as the first day and by improving all outcomes significantly, supporting the contention that toxic blood metabolites are significant in early brain injury [66]. Both ferrous and ferric iron are prooxidant molecules and ROS may promote the transcription of NF-κB and activator protein-1 [67], thus activating inflammatory pathways. ROS production catalyzed by free iron may also cause vasogenic oedema and increase ICP by disrupting BBB [68].

2.8. Small Vessel Spasm. Vascular spasm on angiographic imaging is restricted mostly to proximal large vessels and it occurs 3–7 days after SAH. However evidence from experimental studies shows that the constriction effect seen on parenchymal small vessels within the first minutes after SAH is greater than on large proximal vessels [11, 69]. Technical reasons limit the data on SAH-induced microcirculatory changes to animal studies. They demonstrate the presence of abnormal pial microcirculation with spasm of the microvasculatures, decreased blood flow and agglutination of red blood cells [70]. In the majority of patients, aSAH induces multiple vasospasm of arterioles without angiographic signs of vasospasm or increases in blood flow on evaluation with transcranial Doppler.

Uhl et al. confirmed constriction of small vessels in patients undergoing surgery within the first 72 h after aSAH [71] and they proposed that SAH is associated with a microvascular spasm primarily involving arterioles, with constriction in pial vessels and decrease in capillary density. Pennings later confirmed this finding [72]. In animal and postmortem pathological studies a disruption of the basal membrane and the endothelial layer was demonstrated [73], with morphological changes being more evident on parenchymal vessels compared to large vessels [74]. These may contribute to early clinical signs and may influence the postoperative course [71]. In particular endothelial dysfunction is considered to be one of the key factors initiating early vasoconstriction, keeping in with a decreased response to vasodilators (such as acethilcoline, bradikinine, or thrombin) which require a functional endothelium [75]. Basement membrane degradation seems to be more related to destabilization of microcirculation and increase in vascular permeability and interstitial oedema [76]. Whether these early changes in microcirculation can be used as a prognostic factor for the development of delayed proximal vasospasm remain to be proven.

2.9. Cortical Spreading Depolarization. Cortical spreading depolarization (CSD) is a wave of almost complete depolarization of the neuronal and glial cells that occurs in different neurological diseases [77]. It is observed within the first 72 hours of SAH and it occurs probably as a result of the irritating activity of subarachnoid haemoglobin and an elevated extracellular potassium, glutamate, and ET-1 [78]. This results in a breakdown of ion gradients characterized by a change in the negative potential with an amplitude between −10 and −30 mV and a duration of about one minute. The histological result is neuronal oedema and dendritic distortion. The combination of decreased CBF and increased energy requirements imposed by CSD may worsen neuronal injury [79]. Clustered spreading depolarisations are related to metabolic changes suggestive of ongoing secondary damage primarily in nonischemic brain tissue [80]. Experimentally, spreading depolarization leads to massive increase in glutamate, decrease in glucose, and increase in lactate levels [81]. Under pathologic states of hypoperfusion, cortical spreading depolarization may produce oxidative stress, worsen hypoxia,

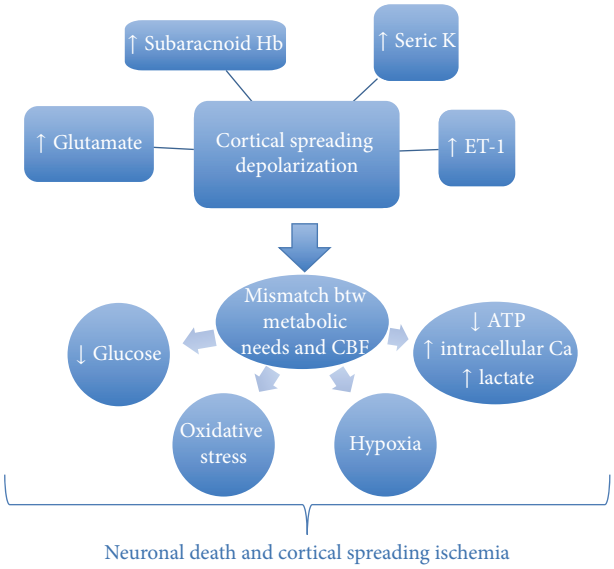

Figure 2

and induce neuronal death [16]. Elevated intracellular calcium is possibly the predominant mediator of neuronal death from ischemia [82] (Figure 2). Clinical studies confirm how the number of spreading depolarisations recorded with a subdural electrode strip correlates significantly with the development of DCI and showed it to be a more reliable marker than vasospasm seen on angiograms [83, 84].

2.10. Cell Death. Secondary brain injury in particular is mediated by apoptosis, while a minor role is exercised by necrosis and autophagy. Cell death starts within 24 hours of SAH, secondary to an early decrease in CPP and CBF with the consequent activation of hypoxia-induced factors and cysteine-aspartic proteases (caspases) [85].

Serum levels of neuron specific enolase, a marker of neuronal injury, show a trend related to the amount of subarachnoid blood, which correlates with poor neurological status on admission [86]. Apoptosis is triggered by elevated ICP, ischemia, reperfusion, and acute vasospasm and by the neurotoxicity of blood breakdown components and oxidative stress [11, 44]. It involves neuronal, glial [87], and smooth muscle and endothelial cells, causing BBB disruption [88] and promoting vasospasm development. A pathological elevation of intracellular calcium activates caspase-dependent apoptotic pathways [89] and beneficial effects have been observed upon inhibition of caspase activity [90] in terms of improvement of cerebral vasospasm in animal models [91]. Furthermore after an interaction between apoptosis and autophagy was demonstrated, rapamycin and simvastatin were shown to inhibit apoptosis by activating post-SAH autophagy [92].

3. Delayed Brain Injury

Many patients survive EBI but deteriorate a few days later after the hemorrhagic onset. The term delayed brain injury (DBI) describes critical events arising in the late phase of aSAH (3–14 days) resulting from the interaction of multiple pathological pathways as a direct consequence of EBI and leading to delayed cerebral ischemia (DCI) [93]. DCI causes poor outcome or death in up to 30% of patients who survive the initial impact of SAH after having their aneurysm treated effectively [94]. DCI is actually thought to be caused by the combined effects of delayed vasospasm, arteriolar constriction, thrombosis and dysfunction in microcirculation, and cortical spreading ischemia, all processes triggered by EBI.

3.1. Delayed Cerebral Vasospasm. Historically delayed spasm in cerebral proximal vessels was thought to be the principal factor responsible for tissue infarction and clinical deterioration and its monitoring was considered a reliable marker in the followup of aSAH patients. Several studies found a correlation between radiologically confirmed vasospasm and clinical symptoms of DCI [95]. In the acute phase it is considered the result of a prolonged contraction of smooth muscle cells, with an abnormal endothelial hypertrophy arising from inflammatory changes and gene expression modification [96]. An increase in inflammatory cells in the adventitia is observed with a necrosis in the muscular layer. In the chronic phase, a proliferation of smooth muscle cells is characteristic, probably mediated by ET-1 [97], finally leading to cerebral ischemia. Vasospasm begins on the third day after the onset of SAH with a peak at 6–8 days, eventually lasting 2-3 weeks [98]. Clinical predictors are volume, density, and prolonged presence of SAH (Fisher classification) [99] and the incidence increases with age and cigarette smoking, preexisting hypertension, and hypovolemia.

A significant relationship between severity of vasospasm and the proportion of patients with infarction was shown by Crowley et al. [100] analyzing the CONSCIOUS-1 data; a strong association was demonstrated between vasospasm seen on angiograms and new cerebral infarctions [101].

The physiopathology of delayed cerebral vasospasm is still poorly understood but many mechanisms are shared with EBI, with activation of inflammatory pathways, oxidative stress, electrolyte changes, and apoptosis activation playing an important role. Vasospasm may critically in fact be a *late* sign of EBI [102] (Figure 3).

3.2. Microcirculation Dysfunction and Vasospasm. Microcirculatory dysfunction is a process distinct from proximal vessel spasm. Normally autoregulation compensates for decreased CPP with a proportional vasodilatation [103]. SAH causes failure of the microcirculation, decrease in the mass density of capillaries and spasm, vasoconstriction, and pathologic changes in small vessels that may lead to infarction. Arteriolar diameter is physiologically the primary determinant for CBF and DIND is likely to be strongly related to microcirculatory changes [9].

Furthermore after aSAH, the coagulation cascade is strongly activated with a diffuse formation of microthrombi. The concentrations of fibrinopeptide A, tissue factor, and thrombin-antithrombin complexes are significantly elevated in patients who developed DCI [104]. The pathological

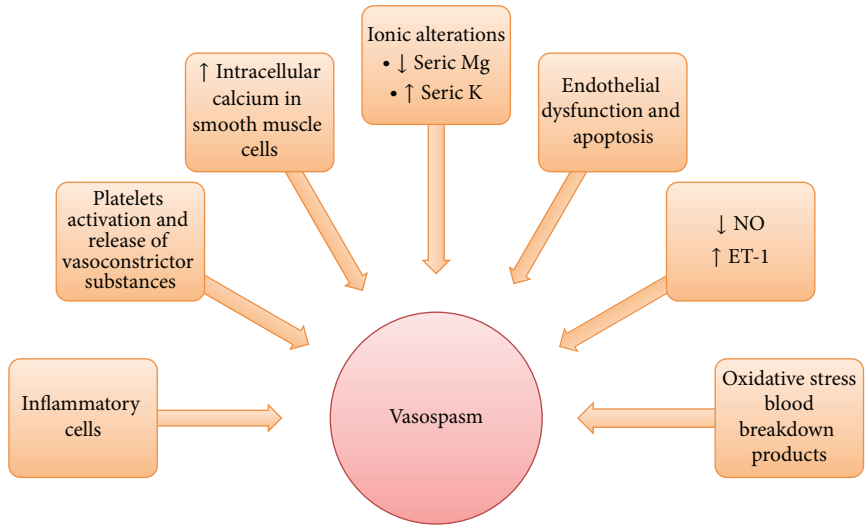

Figure 3

formation of microthrombi blocking the possibility of collateral revascularisaton or causing a persistent "no-reflow" phenomenon may represent an alternative explanation for CBF reduction and DIND, independent of CPP alteration [74].

Using the index of brain tissue oxygen pressure reactivity (ORx, a variable correlation coefficient between cerebral perfusion pressure and partial pressure of brain tissue oxygen), Jaeger et al. showed how impaired autoregulation was associated with an unfavourable outcome in patients with SAH, measured according to their Glasgow Coma Score [105]. Disrupted autoregulation may predict which patients will finally develop delayed infarction [106].

3.3. Cortical Spreading Ischemia. Cortical spreading ischemia is a direct consequence of neuronal/glial depolarization (cerebral spreading depolarization), normally occurring 72 hours after initial haemorrhage. The direct consequence of CSD is consumption of ATP stores, electrolyte imbalance, cerebral oedema, and neuronal death, as a result of a prolonged disproportion between increased metabolic needs and decreased CBF, thus ultimately producing widespread cortical necrosis [107].

4. Therapeutic Strategies

Therapeutic strategies in aSAH are currently designed to treat vasospasm with the ultimate goal of preventing delayed ischemic injury and improving clinical outcome.

4.1. Triple-H Therapy. Triple-H therapy (hypertension, hypervolemia, and hemodilution) was routinely used for prophylaxis and treatment of cerebral vasospasm. Hyperdynamic therapy by increasing blood pressure and, if necessary, cardiac output is considered the best available medical option for treatment of cerebral vasospasm [108, 109]. Raabe et al. showed how moderate perfusion pressure, with a CPP of 80–120 mmHg in a normovolemic hemodiluted patient, is an effective method of improving cerebral autoregulation and is associated with a lower complication rate compared with hypervolemia or aggressive hypertension therapy [110]. Similarly, Muench et al. showed in experimental models how triple-H therapy failed to improve regional blood flow more than maintaining hypertension alone. They showed that the triple-H therapy was characterized by a detrimental effect of hypervolemia and/or hemodilution which reversed the positive effect of induced hypertension on brain tissue oxygenation [111]. In conclusion, hypervolemia and hemodilution are not beneficial on CBF and are not recommended nowadays [112].

4.2. Calcium Channel Blockers. Current SAH treatment protocols include, besides neurointensive care and hyperdynamic therapy, the prophylactic administration of nimodipine. The rationale for the use of calcium antagonists for prevention of secondary ischemia was initially based on the blocking of the dihydropyridine-type calcium channel, thereby preventing the influx of calcium into the vascular smooth-muscle cells and decreasing the rate of cerebral vasospasm [113]. After their introduction into clinical practice it was discovered that calcium-channel blockers have neuroprotective properties and they seem to provide beneficial effects without angiographic evidence of cerebral vasodilatation [113]. Nimodipine, an L-type Ca channel blocker, is currently the only pharmacologic agent showing an improvement in neurological outcome when used for a period of 21 days after aneurysmal rupture. This occurs without a real effect on cerebral vasospasm [114]. Calcium blockers seem useful therapeutic agents outside of any effect on vasospasm [114].

4.3. Magnesium Sulphate. Magnesium sulphate acts as a noncompetitive antagonist of voltage-dependent calcium

channels and as a NMDA-receptor antagonist and has neuroprotective and vasodilator properties [26]. Furthermore magnesium therapy seems to reduce the inflammatory burden in treated patients [115]. Intravenous magnesium sulphate was shown by van den Bergh et al. to reduce cerebral vasospasm and infarct volume after experimental SAH [25]. However, MacDonald et al. [116] and Veyna et al. [117] failed to show prevention or clinical improvement in cerebral vasospasm with magnesium therapy. It has been considered a promising agent [114] but clinical trials failed to demonstrate a clear benefit [118]. Nonetheless, the risk of adverse effects is minimal and some practitioners prefer to maintain high serum levels of magnesium in patients with aSAH.

4.4. ET-1 Receptor Antagonists. Disappointing results were observed in two randomized double blind phase II and III trials using an ET-1 receptor antagonist (clazosentan) [119, 120]: ET-1 seems to have a key role in vasospasm but inhibition of its action seems to reduce cerebral vasospasm without improving final functional outcome or mortality [8]. Only a small reduction was observed in the number of patients exhibiting DIND and there was no beneficial effect on the Glasgow Outcome Scale (GOS) at 3 months' followup. However the sample size estimates for CONSCIOUS-1 trial were not intended to demonstrate an effect on functional outcome and the study was underpowered to detect changes in mortality [119].

The CONSCIOUS-2 trial was designed to investigate whether clazosentan reduced vasospasm-related morbidity and all-cause mortality. It however showed no clinical benefit (including functional outcome) and systemic complications were more frequent in patients treated with this drug [120].

4.5. Vasodilators. Among vasodilator agents, milrinone has the added effect of being an inotrope. It is a phosphodiesterase III inhibitor that increases the level of cyclic adenosine monophosphate (cAMP); it was first used in the short-term therapy for chronic heart failure and its first use in the treatment of cerebral vasospasm after rupture of an intracranial aneurysm was reported in 2001 [121]. Furthermore it has a supposed anti-inflammatory effect [122]. However, in order to obtain a direct effect on EBI, vasodilators should be introduced immediately on hospital admission.

Recent attention was drawn to the vasodilator effects of oestrogen therapy. Ding et al. showed recently how 17 beta-estradiol (E2) is a potent vasodilator [123]. They demonstrated in vivo an attenuated cerebral vasospasm on angiography, which is probably related to a decreased iNOS expression, a normal eNOS expression, and diminished ET-1 production [124]. Furthermore, E2 may have direct antioxidant effects by scavenging ROS and it may decrease TNFα expression through a reduction of JNK signalling activity [125] and of inflammatory pathways. E2 may also inhibit apoptosis by neuroglobin and ERK pathway activation. However, it must be noted that known adverse effects of oestrogen treatment are not negligible.

4.6. Nitric Oxide Donors. NO donors were investigated in experimental studies (sodium nitrate, sodium nitroprusside, and nitrite) and they seem to prevent cerebral vasospasm in a primate model [126]. However, the clinical utility of NO is limited by its short half-life and its potential toxicity [127].

4.7. Antioxidants. Antioxidants such as methylprednisolone (also an anti-inflammatory agent) and tirilazad mesylate (a free radical scavenger) may prevent oxidative stress and EBI damage [128], though apparently with limited efficacy related to a one-year functional outcome [50, 129, 130]. Furthermore, free radical scavengers seem to be associated with a lower incidence of delayed ischemic injury [131].

Recently Zhang et al. [132] published a study of the use of astaxanthin (ATX), one of the most common carotenoids with potent antioxidant properties, on experimental SAH. The authors showed how ATX (by intracerebroventricular injection or oral administration) could significantly alleviate EBI in rat models by reducing brain oedema, BBB disruption, neural cells apoptosis, and neurological dysfunction. ATX may have pleiotropic effects through inhibition of glutamate release [133] by blocking inflammatory pathways (NF-κB) [134], by limiting apoptosis and by platelets aggregation [135]. No side effects were reported following ATX use [136] and it may represent a new promising therapeutic option.

4.8. Nonsteroidal Anti-Inflammatory Agents. Different anti-inflammatory treatments have been studied in cerebral vasospasm with contrasting results. This may be explained by the heterogeneity of inflammatory patterns activated during aSAH [53]. Currently anti-inflammatory agents are not used as standard treatment in patients with SAH. However the use of nonsteroidal anti-inflammatory drugs may produce a reduction in the inflammatory response and reduce the odds for unfavourable outcomes [137].

4.9. Antiplatelet Agents and Inhibitors of Thrombus Formation. Clinical studies using antiplatelet agents show contrasting outcomes; one study showed a reduced risk of cerebral infarction in patients using aspirin [138] and another study showed an increased haemorrhagic volume in patients habitually using cyclooxygenase inhibitors [139].

Cerebrovascular microthrombosis was also the target of therapeutic research; ADAMTS-13 inhibits physiologically thrombus formation and thus inflammatory responses. Its systemic administration in experimental models diminished the microthrombotic process and improved neurological performances probably by limiting neuronal inflammation, without effects on vasospasm [140].

4.10. Statins. The debate is open for the use of statins as a therapeutic option in the acute period after SAH. Statins are hydroxymethylglutaryl- (HMG-) CoA reductase inhibitors with pleiotropic effects; they may decrease the inflammatory burden und upregulate the production of vasodilator substances (NO) by modulating eNOS expression [141, 142]. Furthermore statins may reduce the excitotoxicity of glutamate, inhibit platelet aggregation, and prevent apoptosis

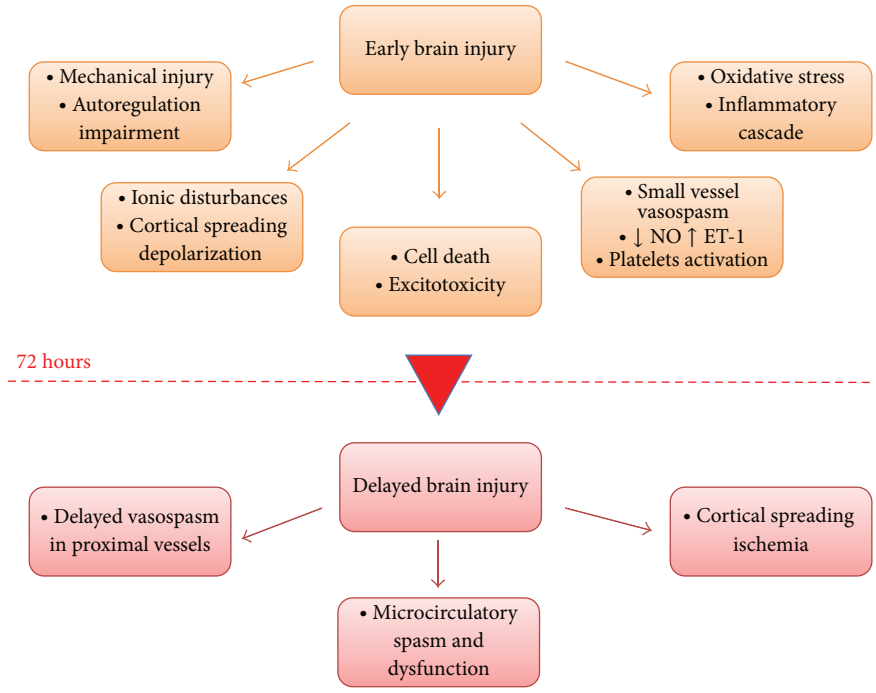

Figure 4

[143]. Some studies showed a decreased incidence of cerebral vasospasm and of mortality rate in patients treated with statins [144, 145]. One meta-analysis showed how statin use decreases the overall incidence of delayed vasospasm, ischemic injury, and mortality [146], while another meta-analysis showed no differences in outcome [147].

5. Discussion

Delayed cerebral vasospasm of proximal cerebral vessels has classically been considered the primary marker to monitor patients' progression [148] and the most important and treatable cause of mortality and morbidity in SAH [149]. In the last few years, the key role of delayed cerebral vasospasm has been questioned; DIND, the principal prognostic determinant in patients surviving the acute phase, has been shown to be a multifactorial process. Multiple mechanisms other than vasospasm may contribute to long-term outcome and the role of events occurring during the immediate hours after bleeding has recently been emphasized (Figure 4).

According to the literature, 21% of aSAH survivors, who do not develop vasospasm, develop delayed ischemic injury and only 20%–30% of those who develop delayed vasospasm suffer from delayed ischemic injury [150]. The cerebral blood flow diminution observed in patients with moderate and even severe vasospasm seems, in fact, not a sufficient cause for cerebral infarction [9]. Some authors claim that many factors determine whether infarction develops after vasospasm confirmed angiographically, including the duration and severity of ischemia, the presence and length of stenosis, and the presence of collateral pathways [151]. In some cases, however, infarction occurs immediately after SAH without detectable vasospasm in the territorial artery [152] and other effects such as microthrombus formation or spasm and dysfunction in the microcirculation may make a significant contribution [153].

Therapeutic strategies in SAH patients are currently still designed to treat vasospasm with the ultimate goal of preventing delayed ischemic injury and improving clinical outcome. These therapies result either in a reduced incidence of radiologically evident vasospasm without improvement in delayed ischemic injury or in quality of life (as in the case of ET-1 receptor antagonists) or in clinical benefit without evident angiographic response in terms of decreased vasospasm (as in the case of calcium blockers).

Different reasons may be put forward to explain these results; bias in the construction of studies could play a role (e.g., sample populations being not big enough to show a real clinical benefit or low sensibility of scores chosen to evaluate clinical outcomes). Furthermore different pathological pathways may be implicated in the final outcome in patients suffering from aSAH, independent of any vasospasm demonstrated angiographically.

The role of vasospasm has probably been misinterpreted; treating vasospasm alone probably targets the wrong focus and may not lead to improvement in functional outcome. The events occurring early after haemorrhage are clearly responsible for the development of delayed ischemia; the massive brain damage observed at autopsy in patients dying within the first 72 hours of haemorrhage confirms the importance of EBI [154]. Acute intracranial circulatory arrest [18] promotes metabolic deregulation and impairment of vascular reactivity resulting in an altered autoregulation and CO_2 responsiveness [155]. Cortical spreading depolarization, inflammation, and oxidative stress may contribute further to

small vessel dysfunction, microthrombosis, and early ischemic signs [156].

Understanding, monitoring, and treating the various mechanisms at the root of early brain injury will be the key to improve the prognosis in SAH. Whilst human data are actually scant, preclinical studies demonstrate that treatment of EBI improves functional outcome.

Examining what happens early on in aSAH is usually monitored in the neurological ICU by a multimodal neuromonitoring [157]. Data existing strongly suggest that biochemical changes detected with cerebral microdialysis may precede the onset of secondary neurological deterioration following SAH [158]. Microdialysis may therefore be a useful tool to optimize neuroreanimation activities based on measures of brain metabolites in extracellular fluid, excitotoxicity and oxidative stress [40] in a the very early phase of SAH. Equally PbtO2 monitoring could help in guiding therapeutic decisions and in predicting the prognosis.

Pharmacological agents able to diminish EBI may include vasodilators (calcium blockers, magnesium sulphate, ET-1 antagonists, NO donors, milrinone, and oestrogen therapy), antioxidants, anti-inflammatory, or antiplatelet agents [13] and iron binding molecules. A combination of these therapeutic options may be necessary to obtain a synergic effect.

New promising strategies include using pleiotropic molecules with vasodilator properties (such as 17β–estradiol), anti-inflammatory and antioxidative drugs, such as astaxanthine or TNFα inhibitors, and molecules limiting microthrombus formation, such as ADAMTS-13. These showed encouraging results in preclinical studies and it is now evident that focusing on vasospasm treatment alone cannot achieve improvement in functional outcome. Promoting strategies to treat early brain injury will prevent many of the tragic consequences of SAH and new therapeutic options should concentrate further research into EBI and consequently DBI determinants [88].

6. Conclusions

Delayed ischemic injury is a complex process, resulting from the contribution of different pathological pathways and it is the leading determinant of poor functional outcome in patients surviving the initial hemorrhagic insult of aSAH. The role of vasospasm has long been overemphasized. Delayed vasospasm is not a necessary prerequisite for DIND development. Vasospasm alone should not be used to monitor the efficacy of therapeutic interventions nor used as a prognostic marker. Indeed, its reversal alone is inadequate as a therapeutic target. Many other mechanisms may underlie prognosis and a contemporary therapeutic approach reacting to multiple pathological pathways evident in early brain injury should be sought. Despite extensive research and aggressive management of cerebral vasospasm (both medical and endovascular), SAH prognosis remains poor. Invasive neuromonitoring to detect pathological alterations occurring in early brain injury may permit prevention of DIND; such therapeutic interventions need to be undertaken within the first hours after aSAH.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Progesterone Attenuates Experimental Subarachnoid Hemorrhage-Induced Vasospasm by Upregulation of Endothelial Nitric Oxide Synthase via Akt Signaling Pathway

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Cerebral vasospasm is the leading cause of mortality and morbidity in patients after aneurysmal subarachnoid hemorrhage (SAH). However, the mechanism and adequate treatment of vasospasm are still elusive. In the present study, we evaluate the effect and possible mechanism of progesterone on SAH-induced vasospasm in a two-hemorrhage rodent model of SAH. Progesterone (8 mg/kg) was subcutaneously injected in ovariectomized female Sprague-Dawley rats one hour after SAH induction. The degree of vasospasm was determined by averaging the cross-sectional areas of basilar artery 7 days after first SAH. Expressions of endothelial nitric oxide synthase (eNOS) and phosphorylated Akt (phospho-Akt) in basilar arteries were evaluated. Prior to perfusion fixation, there were no significant differences among the control and treated groups in physiological parameters recorded. Progesterone treatment significantly ($P < 0.01$) attenuated SAH-induced vasospasm. The SAH-induced suppression of eNOS protein and phospho-Akt were relieved by progesterone treatment. This result further confirmed that progesterone is effective in preventing SAH-induced vasospasm. The beneficial effect of progesterone might be in part related to upregulation of expression of eNOS via Akt signaling pathway after SAH. Progesterone holds therapeutic promise in the treatment of cerebral vasospasm following SAH.

1. Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a serious and fatal disease. The mortality rate is 27% to 44% in SAH patients [1], and 46% of SAH patients survive with serious sequela of cognitive and functional impairment [2]. The main therapy against SAH is securing the cerebral aneurysms and treating the cerebral vasospasm, which develops in 70% of SAH populations between 3 and 14 days after SAH onset [3]. Cerebral vasospasm causes delayed cerebral ischemia and contributes to the major cause of poor outcome and even death in SAH patients [1], but so far there is no definitive treatment against the devastating complication.

Growing evidence shows that progesterone, a sex steroid hormone, attenuates brain edema [4] and has beneficial effects on traumatic brain injury [5, 6], stroke [7, 8], experimental autoimmune encephalomyelitis [9, 10], and experimental spinal cord injury [11].

Our previous studies showed that 17β-estradiol (E2) attenuates SAH-induced vasospasm by the prevention of augmentation of inducible nitric oxide synthase (iNOS) expression and the preservation of normal eNOS expression that reduces secondary brain injury after SAH [12, 13]. The prevention of increased iNOS expression is achieved by interfering with the nuclear factor kappa B transactivation via estrogen receptor-dependent mechanism. Our other findings have also demonstrated that E2 reverses the decreased expression of adenosine A1 receptors and increases the expression of adenosine A2A receptors to prevent vasospasm and apoptosis induced by SAH in the dentate gyrus [14]. E2 treatment may reverse apoptosis by increasing phospho-Akt, ERK, and ERα protein expression in the dentate gyrus via an ERα-dependent pathway [15, 16]. Likewise, progesterone, a sex hormone like E2, has the potential role in treating SAH-induced vasospasm.

Endothelial nitric oxide (NO), enhanced by eNOS, is essential to vascular homeostasis and angiogenesis [17]. And eNOS is activated by various stimulations including the phosphatidylinositol 3-kinase- (PI3K-) protein kinase B/Akt signaling pathway, and phosphorylation of Akt is essential for its activity [18].

In this study, we hypothesized that reversal of spasm of basilar artery after SAH is due to activation of phospho-Akt, which subsequently increases eNOS expression after progesterone treatment. We used two-hemorrhage rodent model of SAH, measured the diameter of basilar artery, and examined the expression of eNOS and phospho-Akt in the basilar artery following SAH.

2. Materials and Methods

2.1. Animals. All experimental protocols were approved by the Kaohsiung Medical University Animal Research Committee. Ovariectomized female Sprague-Dawley rats (NLAC, Education Research Resource, National Laboratory Animal Center, Taiwan), weighing 300–350 gm, were used. The animals were maintained on a 12-hour light/dark cycle, with free access to food and water. Rats were evenly divided into the following four groups. Animals in group 1 served as controls and were not subjected to SAH (control; $n = 20$). The animals in all other groups were subjected to experimental SAH as described below. Group 2 received experimental SAH without additional treatment (SAH only; $n = 20$). Group 3 received experimental SAH plus vehicle (SAH + vehicle; $n = 20$). Group 4 received experimental SAH with progesterone (8 mg/kg/day, subcutaneously) treatment 1 hour after the first induction of SAH (SAH + P; $n = 20$) for 7 days following the first hemorrhage.

2.2. Induction of Experimental SAH. Rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). Animal's head was fixed in stereotactic frame and the cistern magna was punctured percutaneously with a 25-gauge butterfly needle. About 0.1 to 0.15 mL of CSF was slowly withdrawn and the junction of butterfly needle and tube was clamped. Freshly autologous nonheparinized blood (0.3 mL) was withdrawn from tail artery. Using a needle-in-needle method (inserting 30-gauze needle into 25-gauze butterfly needle at the junction of needle and tube), blood was injected slowly into the cistern magna in approximately 2 minutes. The same procedure was repeated 48 hours later. Seven days after the first SAH, animals were sacrificed by perfusion and fixation. Then the brain was removed, placed in a fixative solution, and stored at 4°C overnight. The basilar arteries were isolated for further examination.

2.3. Basilar Artery Morphometric Analysis. Morphometric measurements were performed by an investigator blinded to the treatment groups. At least five random arterial cross-sections from each animal were evaluated qualitatively for the extent of corrugation of the internal elastic lamina (IEL), and the cross-sectional area of each section was measured using a computer-assisted image analysis system. The areas of the five cross-sections from basilar artery were averaged to provide a single value for each animal. Group data were expressed as mean ± SEM. Group comparisons were performed using a one-way analysis of variance (ANOVA). Differences were considered significant at the $P < 0.05$ level.

2.4. Western Blot Analysis. Six animals in each group were included in this protocol. Samples were obtained from the basilar artery (BA). BA tissue was homogenized in ice-cold M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) with Protease inhibitor (Complete Mini; Roche, Mannheim, Germany), centrifuged at 15000 rpm for 20 minutes. The protein concentration was estimated using the Bio-Rad protein microassay procedure. Samples were heated for 5 minutes in boiling water. Equal amounts of protein were loaded in each land of SDS-PAGE. The gels were transferred onto polyvinylidene difluoride (PVDF; PerkinElmer, Waltham, MA) membrane by electroblotting for 90 minutes (100 V), and the membrane was blocked overnight at 4°C with the Tween-Tris buffer saline solution (T-TBS; 20 mM Tris base, 0.44 mM NaCl, 0.1% Tween 20, and pH 7.6) containing 5% nonfat dry milk and 0.1% Tween 20. The blot was incubated with primary antibodies eNOS (1 : 1000; BD), phospho-Akt (1 : 1000; Cell Signaling Technology, Inc., Beverly, MA), Akt (1 : 4000; Cell Signaling Technology, Inc.), and β-actin (1 : 40000; Sigma, St. Louis, MO) and then rinsed with T-TBS for 30 minutes and incubated with goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). Akt and β-actin levels served as an internal standard and to account for loading differences. Membranes were rinsed with T-TBS for 30 minutes, incubated with electrochemiluminescence reagent (PerkinElmer, Waltham, Massachusetts) for 2 minutes, and apposed to the manufacturer's specification. We scanned the X-ray films and the determined optical density by employing ImageJ (National Institutes of Health, Bethesda, MD).

2.5. Statistics. The data were expressed as mean ± standard error of the mean. Differences between the experimental groups were determined with one-way analysis of variance

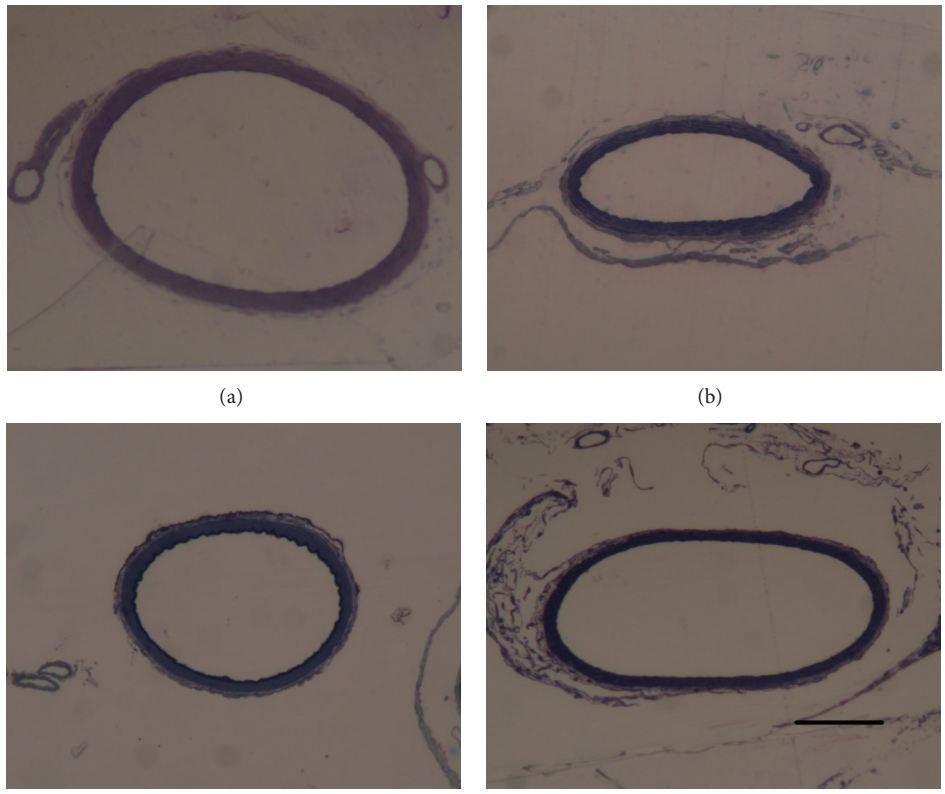

Figure 1: Photomicrographs of representative cross-sections of basilar arteries from control (a), SAH only (b), SAH plus vehicle treatment (c), and SAH plus progesterone treatment (d) (scale bar = 80 μm).

with the Bonferroni post hoc test. Differences were accepted as significant at the $P < 0.05$ level.

3. Results

3.1. General Observations. Prior to perfusion fixation, there were no significant differences among each group of rats in physiological parameters, including body weight, mean arterial blood pressure, and heart rate (data not shown).

3.2. Basilar Artery Cross-Sectional Luminal Area Measurements. The cross-sectional area of basilar arteries was significantly reduced in animals subjected to SAH. Compared with the control group (42255.8 ± 3563.7 μm^2), the areas in the SAH only (25121.5 ± 4361.9 μm^2) and SAH plus vehicle groups (24020.3 ± 3716.1 μm^2) were reduced by 41% ($P < 0.01$) and 43% ($P < 0.01$), respectively (Figures 1 and 2). The cross-sectional areas in the progesterone treatment group (35802.3 ± 3960.5 μm^2) differed significantly from those of the SAH-only group ($P < 0.01$). There was no significant difference between the progesterone treatment group and the control group.

3.3. The Expression of eNOS and Phospho-Akt Proteins in Basilar Artery. The eNOS protein content decreased significantly in the SAH group ($P < 0.01$) and SAH plus vehicle group ($P < 0.05$) when compared with the control group. The eNOS protein content increased significantly in the progesterone treatment group when compared with SAH group ($P < 0.001$) and control group ($P < 0.05$) (Figure 3).

The phospho-Akt protein content decreased significantly in the SAH group ($P < 0.05$) and SAH plus vehicle group ($P < 0.05$) when compared with the control group.

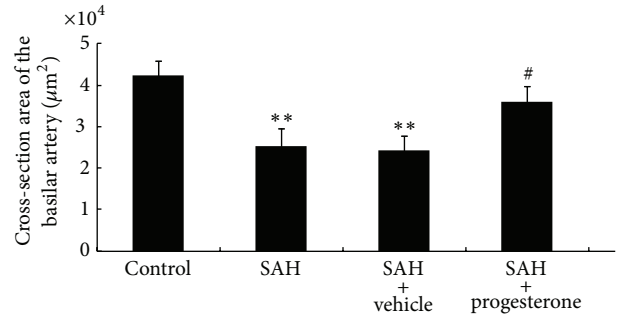

Figure 2: Bar graphs showing the effects of progesterone on cerebral vasospasm in cross-sectional areas. The average luminal area (mean ± SEM) of basilar arteries is demonstrated for each group of animals. Conversely, the basilar artery luminal areas of the progesterone treated group were markedly larger than those in the SAH-only group. $^{\#}P < 0.05$ compared with the SAH-only group; $^{**}P < 0.01$, significantly different from the control group.

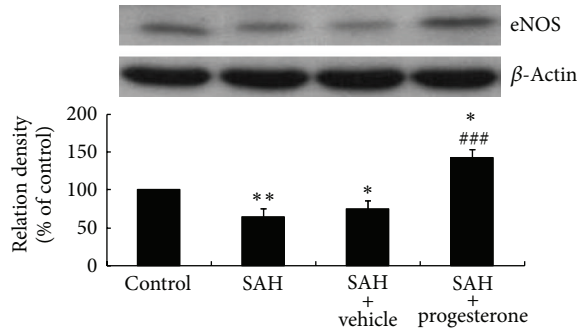

FIGURE 3: Bar graphs showing progesterone treatment inducing the increase in eNOS protein expression within the basilar artery following SAH. $^{\#}P < 0.05$, significantly different from the SAH group; $^{\#\#\#}P < 0.001$, significantly different from the SAH group; $^{*}P < 0.05$, significantly different from the control group; $^{**}P < 0.01$, significantly different from the control group.

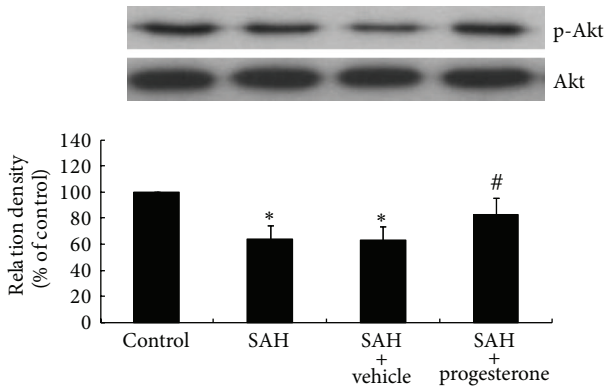

FIGURE 4: Bar graphs showing progesterone treatment enhancing phospho-Akt protein expression within the basilar artery following SAH. $^{\#}P < 0.05$, significantly different from the SAH group; $^{*}P < 0.05$, significantly different from the control group.

The phospho-Akt protein content increased significantly in the progesterone treatment group when compared with the SAH group ($P < 0.05$). No significant difference of phospho-Akt protein amount was observed between the progesterone treatment group and control group (Figure 4).

4. Discussion

Treatments for cerebral aneurysms and associated vasospasm are complicated. Besides, patients with vasospasm have more inpatient costs and longer hospital stays [19]. Presently, no definite medical treatment is effective against vasospasm. Clinically we use oral nimodipine for patients with SAH, but nimodipine improves functional outcome not contributing to the improvement of vasospasm but contributing to its neuroprotective effect [20]. Recently, a phase 3 randomized trial, MASH-2, published its results that magnesium sulfate does not improve clinical outcome in 1,203 patients with aneurysmal SAH [21]. Progesterone has been proven to show beneficial effects on traumatic brain injury and stroke in experimental models. O'Connor and colleagues showed that progesterone decreased the apoptosis in hippocampus and cortex and improved the motor performance after diffuse traumatic brain injury in rats [5]. Wang and colleagues demonstrated that progesterone reduced the infraction volume, decreased brain edema, and improved functional outcome in middle cerebral artery occlusion rats [7]. Our study showed that progesterone has therapeutic effects in SAH-induced vasospasm.

Currently, few studies have investigated the role of progesterone in SAH or SAH-induced vasospasm. One study from Yan et al. showed that progesterone decreased brain water content, restored blood-brain barrier, and decreased expression of MMP-9 and caspase-3 in rats within 24 hours after SAH onset [22], which demonstrated that progesterone had neuroprotective effects mainly in early brain injury after SAH. To our knowledge, our study is the first study to apply progesterone in the treatment of SAH-induced vasospasm, which causes delayed brain injury.

In the present study, the expression of eNOS decreased markedly after SAH and treatment with progesterone enhanced the expression of eNOS and reversed the vasospasm of basilar arteries. Khurana et al. demonstrated that expression of recombinant eNOS in the BA relieved SAH-induced vasospasm [23]. In our laboratory, increased expression of eNOS mRNA was also proven to prevent SAH-induced vasospasm via treatment of E2 in experimental models [12]. In addition to SAH-induced vasospasm, NO produced by eNOS elicits vasodilation and consequent neuroprotective effects after brain ischemia [24]. NO is a potent vasodilator and in the condition of vasospasm, any treatment related to eNOS upregulation and NO production would be a promising therapy. In addition to cerebral arteries, progesterone was also shown to increase NO production by upregulating expression of eNOS in endothelial cells of rat aortas [25] and human umbilical veins [26]. Accordingly, progesterone has an important role in activating eNOS in vascular endothelial cells.

NO is essential for vascular tone regulation and it is induced by eNOS, which is stimulated by blood shear stress at endothelial cells [27]. And Dimmeler et al. demonstrated that Akt phosphorylated the Serine 1177 site of eNOS protein and that enhanced eNOS activity; they inhibited the PI3K/Akt pathway and that led to prevention of eNOS activation [28], and Fulton et al. had similar findings [29]. These findings showed that Akt plays a crucial role in eNOS activity and this molecular mechanism is important in many ways, including endothelial cell migration and angiogenesis [30], E2-induced vasodilation [31], protection in ventilator-associated lung injury [32], and protection in intestinal tissue in the situation of intestinal ischemia by Akt-dependent activation of endothelial nitric oxide synthase and vasodilation [33]. Our previous studies showed that E2 attenuates SAH-induced vasospasm by the preservation of normal eNOS expression and reduces secondary brain injury by increasing phospho-Akt, ERK, and ERα protein expression in the dentate gyrus after SAH [12, 13, 15, 16]. And in this study, we suggest that progesterone also showed its antivasospasm effect in this pathway.

It has been shown that progesterone elicited neuroprotective effects in the brain via phosphorylation of Akt [34]. Furthermore, progesterone was also proven to decrease apoptosis via PI3K/Akt pathway in ischemic brain injury [8]. In addition, similar to our results, Khorram and Han demonstrated that progesterone stimulated the production of eNOS protein in human endometrial-derived epithelial cells, and the effect was inhibited completely by an inhibitor of PI3K/Akt pathway called wortmannin [35]. So these results strongly suggest that the effect of progesterone on eNOS was mediated via PI3K/Akt pathway.

5. Conclusion

Treatment with progesterone is effective in the prevention of vasospasm in a two-hemorrhage rodent model of SAH. The antivasospasm effect of progesterone may arise from increased expression of eNOS via the PI3K/Akt pathway after SAH. The use of progesterone is promising in the treatment of cerebral vasospasm following aneurysmal SAH and needs further investigation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of Arterioles and the Microcirculation in the Development of Vasospasm after Aneurysmal SAH

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Cerebral vasospasm of the major cerebral arteries, which is characterized by angiographic narrowing of those vessels, had been recognized as a main contributor to delayed cerebral ischemia (DCI) in subarachnoid hemorrhage (SAH) patients. However, the CONSCIOUS-1 trial revealed that clazosentan could not improve mortality or clinical outcome in spite of successful reduction of relative risk in angiographic vasospasm. This result indicates that the pathophysiology underlying DCI is multifactorial and that other pathophysiological factors, which are independent of angiographic vasospasm, can contribute to the outcome. Recent studies have focused on microcirculatory disturbance, such as microthrombosis and arteriolar constriction, as a factor affecting cerebral ischemia after SAH. Reports detecting microthrombosis and arteriolar constriction will be reviewed, and the role of the microcirculation on cerebral ischemia during vasospasm after SAH will be discussed.

1. Introduction

Cerebral vasospasm after aneurysmal SAH was demonstrated for the first time by Ecker and Riemenschneider in 1951 [1]. They indicated that arterial narrowing of the major arteries near the circle of Willis could be seen on cerebral angiography in six of 29 cases of SAH. Since then, cerebral vasospasm of the major cerebral arteries has been recognized as a main contributor to delayed neurological deterioration of SAH patients, and this deterioration is referred to as delayed ischemic neurological deficits (DIND) or delayed cerebral ischemia (DCI). A large number of investigations have been carried out in an attempt to clarify the mechanism of sustained constriction of arterial smooth muscle cells and to develop treatment methods to ameliorate it. In contrast, there has been little attention paid to cerebral microcirculation as a factor affecting DCI.

However, the CONSCIOUS-1 trial (use of clazosentan to overcome neurological ischemia and infarction occurring after SAH), which was a randomized, blinded clinical trial using an endothelin antagonist, clazosentan, revealed that clazosentan could not improve mortality or clinical outcome in spite of a successful reduction of relative risk of angiographic vasospasm by 65% [2]. This result indicates that the pathophysiology underlying DCI is multifactorial and that other pathophysiological factors, which are independent of angiographic vasospasm, can contribute to the outcome [3]. Recently, the focus has shifted to cerebral microcirculatory disturbance, a pathophysiological factor other than vasospasm of the major cerebral arteries that was not considered important in the past. The trend toward studying the microcirculation during cerebral vasospasm, as it emerged in the past and presently, is reviewed and the significance of microcirculatory disturbance is discussed.

2. Narrowing of the Large Cerebral Arteries and Cerebral Ischemia

The first step in establishing the concept of cerebral vasospasm was based on the recognizing that the narrowing of the major cerebral arteries correlates well with cerebral ischemia. As an early study, Fletcher et al. found that angiographic vasospasm relates to poor neurologic status and focal

neurologic deficits. Angiographic vasospasm was described as segmental or diffused and found to be present three weeks after SAH in 39 of 100 patients [4]. Fisher et al. graded angiographic vasospasm from grades 0 to IV according to the diameter of the residual lumen of the proximal segments of the anterior and middle cerebral arteries. Of 31 patients with grade III or grade IV, 80% of cases developed DIND. Of 19 patients with a lesser grade, none developed DIND [3]. Saito et al. reported that angiographic vasospasm was sometimes correlated with neurological signs and symptoms, while cases with no neurological deterioration exhibited only slight angiographic vasospasm. They classified angiographic vasospasm as extensive diffuse, multisegmental, or local and indicated that the mortality rates associated with these types were 45, 19, and 10%, respectively [5]. Weir et al. measured eight arterial points on 627 angiograms from 293 patients and indicated that the patients with the most angiographic vasospasm had significantly higher mortality rates than those with the least angiographic vasospasm [6].

Thus, most studies before 1980 had pointed to an association of the degree of angiographic vasospasm with neurological impairments or patient outcome. In addition, a correlation was indicated between angiographic vasospasm and cerebral blood flow (CBF) detected by SPECT and emission CT with 133Xe inhalation [7, 8]. These findings suggested that luminal decrease of the major cerebral arteries is the main factor causing reduced CBF and ischemic symptoms.

3. Previous Concepts regarding DCI Pathogenesis

Novel pathological mechanisms have been suggested, including damage to cerebral tissue in the first 72 hours after aneurysm rupture (early brain injury), cortical spreading depression (CSD), microcirculatory dysfunction, and microthrombosis [9–12].

3.1. Early Brain Injury. Early brain injury is a term that refers to the damage done to the brain in the first 72 hours after the initial bleeding [13]. The release of arterial blood into the subarachnoid space is accompanied by intense headache and an acute increase in intracranial pressure, often causing intracranial circulatory arrest and loss of consciousness [14, 15]. The mechanisms of the resulting early brain injury are dominated by cell death, blood-brain barrier (BBB) disruption, and brain edema [16]. Animal models show BBB disruption as early as 30 minutes after cortical SAH [17], and the leakage of large molecules remains high within the first 48 hours of bleeding [18]. It seems probable that the physiological changes occurring at the onset of DCI directly influence the severity of later ischemic complications in patients after SAH. Although experimental results done mainly in rats seem to mirror measurements in patients with aneurysmal rebleeding and intracranial pressure (ICP) monitoring in situ, the majority of animal models rely on iatrogenic damage to cerebral vessels to simulate aneurysm rupture and induce subarachnoid bleeding [19].

3.2. Cortical Spreading Depression (CSD) and DCI. The first CSD was demonstrated experimentally in the 1940s in rabbit cortex. Originally, the process was regarded as an experimental artifact that had little relevance to neurological disease in humans. Recently, however, there has been a resurgence of interest in CSD. In the last few years, CSD has been identified as a potential pathophysiological mechanism contributing to DCI. The term describes a depolarization wave in cerebral grey matter that propagates across the brain at 2–5 mm/min and results in depression of evoked and spontaneous EEG activity [20]. It has been implicated in the pathophysiology of a number of neurological diseases, including malignant hemispheric stroke [21], traumatic brain injury [22], and DCI after SAH [23]. In SAH, there is good evidence from animal models and patient studies that CSDs occur after the initial bleed [23, 24]. Spreading ischemia was first described in a rat model of DCI and it results from local microvascular dysfunction. It is thought that, with each depolarization, there is an associated, profound hypoperfusion of the cortex due to vasoconstriction [25].

The incidence of CSDs measured in patients after SAH seems to correlate with the time frame for the development of DCI, with data from one study demonstrating that 75% of all CSDs recorded occurred between the fifth and seventh days after SAH [26]. CSDs also seem to occur in the absence of angiographic vasoconstriction. Despite placement of nicardipine pellets around the middle cerebral artery to minimize proximal vasoconstriction, spontaneous depolarizations still occurred in 10 of 13 patients [27], casting further doubt on the exact nature of the contribution of proximal vessel constriction to DCI.

3.3. Microcirculatory Dysfunction and Microthrombosis. Clinical investigations had suggested that intraparenchymal small vessels are dilated after SAH in order to compensate for reduced peripheral perfusion pressure caused by vasospasm of the major arteries. Grubb et al. investigated CBF, CMRO2, and cerebral blood volume (CBV) by using positron emission tomography (PET) in SAH patients. A decrease in CBF and CMRO2 and an increase in CBV were seen in patients with poor-grade SAH and severe symptomatic vasospasm. The poor grade patients with symptomatic vasospasm showed reduced CBF under 20 mL/100 g/min and increased CBV over 2 mL/100 g/min. They suggested that cerebral vasospasm of large arteries is accompanied by a massive dilation of the intraparenchymal vessels [28]. A CBF study using single photon emission computed tomography showed that CBF did not increase by administering acetazolamide in SAH patients [29]. Furthermore, a transcranial Doppler sonography (TCD) study revealed that hypercapnia does not decrease flow velocities in the middle cerebral arteries or the internal carotid arteries of patients with cerebral vasospasm [30]. These decreased reactivities to vasodilating stimuli were thought to be due to a lack of response by small vessels because of their maximal dilation in an attempt to maintain sufficient CBF in the face of severe vasospasm [29, 30]. Studies using several imaging techniques in patients with SAH [31–34] and in animal models of SAH [35–37]

have suggested the existence of microvessel constriction and microthrombi formation after SAH. In clinical studies, large artery angiographic vasospasm on admission angiography is also an adverse prognostic factor for outcome [38, 39]. One limitation of these imaging studies is that they examined microvessels visible on the pial surface. Activation of the coagulation cascade, impairment of the fibrinolytic cascade, activation of inflammation, and endothelium related processes may all play a role [40]. Histological studies on a prechiasmatic injection SAH model showed that microthromboemboli are abundant in brain parenchyma [41–43]. Sehba et al. demonstrated the involvement of platelet aggregation and neutrophil infiltration of the observed microvascular injury in the vascular perforation model [44, 45].

In the main, the concept that small vessels dilate during cerebral vasospasm was considered to be valid, and several reports that showed microthrombosis or narrowing of small vessels, as described later, were not considered significant.

Postmortem studies of SAH patients have demonstrated evidence of microthrombi. Patients with DCI have significantly more microthrombi in areas showing cerebral infarction than those patients who die from aneurysmal rebleed or hydrocephalus [46]. Microthrombosis also correlated with the amount of overlying free subarachnoid blood and clinical and pathological signs of ischemia [47]. Interestingly, a postmortem study into microthrombosis after SAH showed that, while cortical ischemic lesions were present in 77% of patients, there was no significant association between the presence of these lesions and angiographic vasospasm or aneurysm location [48].

4. Reassessment of the Role of the Microcirculation in Cerebral Ischemia during Vasospasm

During the period when the concepts described above prevailed, several reports indicated a discrepancy between the degree of angiographic vasospasm and DCI or decreased CBF. There had been several reports showing that clinical symptoms of DCI or decreased CBF can occur without angiographic evidence of vasospasm [8, 49, 50] and that severe angiographic vasospasm is often found in the patients without obvious DCI or decreased CBF [51–53]. However, these concepts were not considered important before the 2000s.

Recently, reports showing that angiographic vasospasm is not always correlated with DCI, cerebral infarction, or CBF have been accumulating. Rabinstein et al. indicated that the location of cerebral infarction in SAH patients cannot be predicted in one-quarter to one-third of patients by angiogram or TCD [54]. Weidauer et al. revealed that cortical band-like infarction develops without evidence of severe angiographic vasospasm in SAH patients [55, 56]. And some clinical studies indicated that angiographic vasospasm of large arteries on admission is an adverse prognostic factor for outcome [38, 39].

Dissociation between angiographic vasospasm and outcome after SAH was noted in the CONSCIOUS-1 trial, which became epoch making in terms of changing the concept of vasospasm [2]. After the CONSCIOUS-1 trial, reports of a discrepancy between angiographic vasospasm and CT hypodensities or regional hypoperfusion have been increasing [57, 58]. One of the explanations for these observations could be that the cerebral microcirculation and its regulatory mechanisms are directly affected by SAH and cause DCI. For microcirculatory dysfunction during vasospasm after SAH, mainly microthrombosis and microarterial constriction have been investigated [12, 59].

5. Microthrombosis

5.1. Detection of Microthrombi. Adhesion of aggregated platelets or mural thrombi at the site of vasospasm of major cerebral arteries had been indicated in early reports [60–62]. Reports on the action of these platelets and thrombi suggest that arteries are narrowed by mural thrombi as well as by proliferative organic changes in the arterial wall and such narrowing is often confused with prolonged vasospasm [62, 63]. Also, aggregated platelets may release vasoactive substances that produce smooth muscle constriction that results in arterial narrowing [60].

Microthrombi were detected for the first time in 1983 by Suzuki et al. in a patient who died due to cerebral vasospasm after SAH. Light microscopy of sectioned slices showed that the parenchymal microthrombi consisted mostly of white thrombi composed of aggregated platelets, with fibrin also observed in some of these. They suggested that microthrombi could be contributors to cerebral ischemia during vasospasm [64]. Later, they confirmed the role of microthrombi in cerebral vasospasm by investigating six patients who died after SAH. Of the six patients, four died of DCI and two of rebleeding or acute hydrocephalus. Compared to the latter two patients, the other four patients showed significantly more microthrombi of intraparenchymal small vessels in clinically ischemic regions and in areas showing cerebral infarction on CT scan. They concluded that the significant regional correlation of thrombi distribution and DCI suggests a close relationship between them [46]. This is supported by a recent autopsy study investigating 29 SAH patients by Stein et al. They revealed a strong correlation between microclot burden and DCI, as patients with clinical or radiological evidence of DCI had, in the mean, significantly more microclot burdens than patients without DCI. And there was also a significant association between microclot burden and histological evidence of ischemia [47].

Furthermore, microthrombi have also been shown in experimental studies. Seven days after SAH, microthrombi in the cerebral and cerebellar cortex can be found in the rat SAH model, in which SAH is produced by blood injection into the prechiasmatic cistern [65]. Peak microthrombi formation is seen 48 hours after SAH in an endovascular perforation SAH model of mouse [66]. Microthrombi in the parenchymal arterioles are also seen 48 hours after SAH in a prechiasmatic blood injection model in mice [12]. In vivo fluorescence

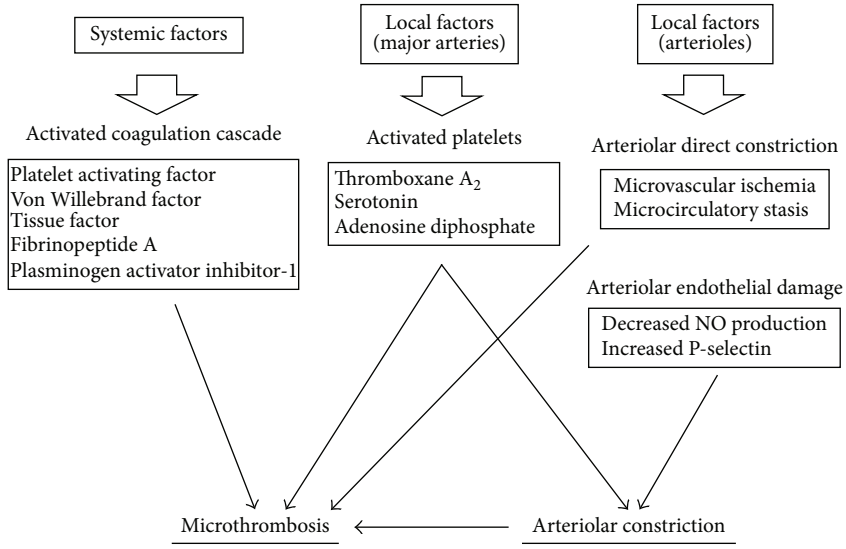

Figure 1: Pathophysiology and mechanism of microthrombus formation and arteriolar constriction.

microscopy using a mouse endovascular perforation SAH model revealed that 30% of pial arterioles were occluded by microthrombi, which demonstrates that microthrombosis is not a histological artifact but also occurs after SAH in vivo [37]. Accumulation of this clinical and experimental evidence seems to strongly affirm the concept that microthrombi comprise one important factor affecting cerebral ischemia after SAH.

5.2. Pathophysiology and Mechanism of Microthrombus Formation (Figure 1). Endothelial function of major cerebral arteries is known to be disturbed during vasospasm after SAH [67]. Prostaglandin I2 is synthesized in the endothelial cells and inhibits the circulating platelets from adhering and aggregating to endothelial cells, and its synthesis at the major cerebral arteries is disturbed during vasospasm [68].

Ohkuma et al. revealed that antiplatelet-aggregating activity in the endothelial cells of the basilar artery is impaired in feline two-hemorrhage SAH models. After production of SAH, adenosine diphosphate was infused into the basilar artery via the right vertebral artery to activate circulating platelets,and many platelets were observed adhering or aggregating on the luminal surface four to seven days after SAH. They suggested that this impairment may be involved in inducing cerebral ischemia during cerebral vasospasm by causing platelet adhesion and aggregation [69]. They also indicated increased platelet function in the case of DCI. Sequential changes of platelet aggregability and beta-thromboglobulin and thromboxane B2 concentrations in blood samples from the internal jugular and peripheral vein were investigated, and platelet function in patients with symptomatic vasospasm showed more enhancements in blood from the internal jugular vein than in blood from a peripheral vein. These results suggest that platelets are activated through vasospastic major arteries and that the resulting increased tendency for thrombus formation may affect the patient's prognosis during the advanced stage [70].

Some reports suggest that a functional disturbance of microvessels itself can be a causative factor for microthrombi. Sabri et al. indicated that decreased NO and increased P-selectin in the endothelium of arterioles is a mechanism for microthrombosis [12]. Direct observation of pial arterioles after endovascular perforation in an SAH model in mice indicated that arteriolar constriction is followed by local formation of microthrombi, and the frequency of arteriolar microthrombosis correlates with the degree of its constriction [37]. This finding also suggests that functional damage in arterioles can cause local thrombus formation.

Changes in the blood coagulation cascade can also contribute to microthrombi formation [40]. Several studies show that the coagulation cascade is already activated within a few days after SAH before the occurrence of vasospasm. This early activation of the coagulation pathway is an early predictor of the occurrence of DCI and infarction after SAH [40]. Levels of platelet activating factor in internal jugular venous blood start to increase within four days after SAH [71]. Increased levels of Von Willebrand factor within 72 hours after SAH correlate with the occurrence of DCI and poor outcome after SAH [72]. These factors are considered to induce platelet activation in the early stage of SAH [40]. Furthermore, in the acute phase after SAH, concentrations of tissue factor, which is the primary initiator of coagulation through activating thrombin, are elevated in the cerebrospinal fluid (CSF) [73].

Increased levels of fibrinopeptide A, an alternative marker of thrombin generation, within two days after SAH, are also associated with cerebral infarction after SAH [74]. And patients with DCI after SAH have significantly higher levels of plasminogen activator inhibitor-1 antigen in the CSF as compared with patients without DCI, suggesting that overactive inhibition of fibrinolysis is associated with DCI [74]. Fibrin formation then also takes part in increased coagulation activity. Therefore, a systemic coagulation cascade activated in the early stage, endothelial dysfunction of microvessels, and platelet activation through vasospastic large arteries are,

together, involved in microthrombi formation and its effect DCI [75].

5.3. Prevention of Microthrombosis. Trapidil, an antagonist and selective synthesis inhibitor of thromboxane A_2, was administrated in a series of 20 cases of SAH. Vasospasm was demonstrated by angiography in nine of these cases, but only two of the nine showed mild signs of cerebral ischemia, which suggests the significance in symptomatic vasospasm of thrombus formation by platelet aggregation and the effectiveness of trapidil as a preventive agent [76]. They also tried OKY-046, an imidazole derivative and a thromboxane syntheses inhibitor; it was studied cooperatively at ten neurosurgical services [77] or at 48 neurosurgical services in Japan in double-blind fashion [78]. Both trials showed the usefulness of OKY-046 for the prevention of symptomatic vasospasm and support the hypothesis that cerebral microthrombosis plays an important role in the pathogenesis of cerebral vasospasm.

Aspirin was used in two trials; both studies, however, included a small number of patients and failed to show its efficacy for the prevention of symptomatic vasospasm or improved outcome [79, 80]. Dipyridamole also failed to show efficacy for reducing the incidence of ischemic deficits [81]. However, a systematic review including five studies indicates that antiplatelet drugs reduce the risk of DCI in patients with SAH [82]. Recent experimental studies showed simvastatin and mutant thrombin-activated urokinase-type plasminogen activator are effective in reducing microthrombi [65, 66]. Recent clinical studies have also indicated that cilostazol, which is a selective inhibitor of phosphodiesterase 3, an antiplatelet agent marketed in Japan, and which is used to treat ischemic symptoms of peripheral vascular disease, can decrease the incidence of symptomatic vasospasm, severe angiographic vasospasm, vasospasm-related new cerebral infarctions, and poor outcome in patients with aneurysmal SAH [83–85].

6. Vasoconstriction of Arterioles

6.1. Detection of Arteriolar Constriction. Microvessel constriction after SAH was demonstrated by Herz et al. in a vascular micropuncture model of SAH in guinea pigs. Their experiments also suggested that a chemomechanical mechanism might be involved in the vasoconstriction of pial microvessels [35]. However, the observation period of microvessel constriction was limited to the unlra-early stage after SAH or stimulation. Hart performed morphometric determinations of the external diameter and wall thickness of intraparenchymal arterioles two hours after blood was injected into the cisterna magna of cats and observed a decreased external arteriolar diameter accompanied by an increased wall thickness. He suggested these changes were caused by constriction of arterioles [86]. However, serial morphological changes in the intraparenchymal arterioles days after SAH coincident with delayed cerebral vasospasm of large cerebral arteries remain unclear in those investigations.

Morphological changes of parenchymal arterioles during the vasospasm period after SAH were examined for the first time by Ohkuma et al. In a canine double hemorrhage SAH model, corrosion casts of arterioles showed tapered narrowing with folding after SAH. Morphometric examination by light microscopy showed a significant decrease in the internal diameter of arterioles associated with a significant increase in wall thickness three and seven days after SAH. These results suggest that constriction of intraparenchymal arterioles occurs after SAH and may contribute to delayed cerebral ischemia [87]. They also revealed that the same changes in perforating arteries occurred in basilar arteries by using the same technique and the same animal model [88].

After that, pial microvessels were directly observed under an operative microscope during aneurysm surgery. Uhl et al. examined pial microcirculation in humans using orthogonal polarization spectral imaging. Patients with SAH who were operated on within three days after SAH showed that capillary density was significantly decreased and small arteries and arterioles of the cortical surface exhibited vasospasm. They suggested those changes may contribute to the initial clinical symptoms and may have an influence on the clinical postoperative course [32]. By using the same technology, Pennings et al. tested contractile responses of the cerebral arterioles in 16 patients who underwent aneurysm surgery. Ten patients were operated on early (within 48 hours after bleeding) and six underwent late surgery. The contractile response of the arterioles to hyperventilation was increased, accompanied by a bead-string constriction pattern in patients operated on early compared to those in late surgeries and in controls [33]. They also revealed the microvascular responses to papaverine in patients undergoing aneurysm surgery. In patients with SAH, unpredictable response patterns to papaverine were observed with "rebound" vasoconstriction. They considered that the results suggest increased contractility of the microcirculation [34].

In addition, recent experimental studies showed the same results. Friedrich et al. examined pial arterioles three, six, and 72 hours after SAH by using in vivo fluorescence microscopy in an endovascular perforation SAH model of mice and found that arterioles constricted by 22% to 33% up to three days after SAH, which demonstrates that SAH induces microarterial constrictions and microthrombosis in vivo [37]. They suggested that these findings may explain the early cerebral perfusion pressure-independent decrease in CBF after SAH and therefore microarterial constrictions and microthrombosis may serve as novel targets for the treatment of early perfusion deficits after SAH.

6.2. Pathophysiology and Mechanism of Microvessel Constriction (Figure 1). Arterioles can constrict in response to various vasoactive substances [89]. Spasmogenic substances derived from subarachnoid clots can then easily affect pial arterioles. And they also affect intraparenchymal arterioles by penetrating into the perivascular space [87]. Another possible factor is vasoactive substances that act on the wall of arterioles from inside the vessels. Circulating platelets activated by endothelial dysfunction through the vasospastic large cerebral arteries liberate vasospastic substances, such as thromboxane A_2, serotonin, or adenosine diphosphate, which can induce smooth muscle constriction in peripheral

arterioles [70]. Endothelial dysfunction of arterioles, such as decreased NO production, can cause arteriolar constriction [12].

Arteriolar constriction is now believed to play an important role in DCI after SAH, but there are several problems to be solved. Microvessel constriction during the period of vasospasm has not been fully proved in humans. Ohkuma et al. measured cerebral circulation time (CCT) and CBF in 24 cases of aneurysmal SAH. CCT was divided into proximal CCT, which was the circulation time through the extraparenchymal large arteries, and peripheral CCT, which was the circulation time through the intraparenchymal small vessels. Peripheral CCT showed a strong inverse correlation with rCBF. Even in nonmild or moderate angiographical vasospasm, prolonged peripheral CCT was clearly associated with decreased rCBF [90]. In other words, rCBF decreased in spite of the absence of angiographical vasospasm in the major artery. These results suggested that microvessel constriction prolonged peripheral CCT and decreased rCBF independently. It was also considered that the cause of peripheral CCT prolongation was based not only on microvessel constriction but also on a microthrombosis.

As far as other problems to be solved, we still need clarification on how many vessels are affected, which microvessels are mostly affected, and how much microvessel constriction is associated with cerebral ischemia. Those problems should be addressed in the future in order to improve outcome for SAH patients by establishing prevention and treatment of arteriolar constriction.

Conflict of Interests

The authors declare that they have no conflict of interests concerning this paper.

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Clinical Study

Prophylactic Intra-Arterial Injection of Vasodilator for Asymptomatic Vasospasm Converts the Patient to Symptomatic Vasospasm due to Severe Microcirculatory Imbalance

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Object. The strategy to treat asymptomatic angiographic vasospasm following subarachnoid hemorrhage (SAH) is controversial. In this study we review our consecutive vasospasm series and discuss an adequate treatment strategy for asymptomatic vasospasm. *Methods*. From January 2007 to December 2012 we treated 281 aneurysmal SAH cases, with postoperative angiography performed 9 ± 2 days after the onset of SAH. Four asymptomatic cases received intra-arterial (IA) injection of vasodilator due to angiographic vasospasm. All cases improved vasospasm immediately following intervention. But all cases turned symptomatic within 48 hours. We retrospectively analyzed the time-density angiography curve and calculated the time to peak (TTP), mean transit time (MTT), and relative blood flow (rBF). Relative blood flow was calculated as follows. The integration of the value of the time-density curve for the artery was divided by the same value for the internal carotid artery multiplied by the MTT. *Results*. The decrease in TTP and MTT for the etiologic artery was similar to that of the nonetiologic artery. But the improvement in rBF for the etiologic artery and nonetiologic artery was 10% and 17%, respectively. Blood supply to the spastic artery decreased due to iatrogenic steal. *Conclusion*. Prophylactic IA injection of vasodilator in cases of asymptomatic vasospasm can produce symptomatic vasospasm.

1. Introduction

Aneurysmal subarachnoid hemorrhage (SAH) impacts approximately 10–21 in 100,000 individuals annually [1–3]. In an aging society, the rupture rate for aged persons is relatively high and can be expected to increase [4–6]. Overall mortality and morbidity are greater than 50% [4, 7, 8]. A major influence on the outcome of postoperated SAH patients is cerebral vasospasm (CVS) [6, 9, 10]. Many factors contribute to the development of CVS: distal microcirculatory failure, poor collateral anatomy, genetic or physiological variations, cortical spread of depolarization, and remodeling [11]. Even when aggressive treatment of CVS was initiated, the reported incidence of symptomatic CVS still ranged from 10 to 50% [6, 12–15]. Intra-arterial (IA) injection of papaverine, nimodipine, nicardipine, fasudil, and milrinone dilates the CVS artery [16–20]. Such IA therapies are effective for symptomatic cases. Prophylactic transluminal balloon angioplasty within 96 hours after SAH also significantly reduces the need for therapeutic angioplasty, but this does not improve poor patient outcome [21].

Recent advances in the noninvasive investigation of cerebral blood flow have led to improved detection of the progression of CVS [22, 23]. The gold standard for the detection of CVS is cerebral digital subtraction angiography (DSA), because this modality measures cerebral circulation time and results in prompt intervention for CVS [24]. Ohkuma et al. detected that peripheral cerebral circulation time was negatively correlated with regional cerebral blood flow using DSA and single photon emission CT in cases of SAH [25]. But the strategy to treat asymptomatic angiographic vasospasm is controversial. We monitored four consecutive asymptomatic cases of angiographic vasospasm that changed to symptomatic CVS within 48 hours after the IA injection

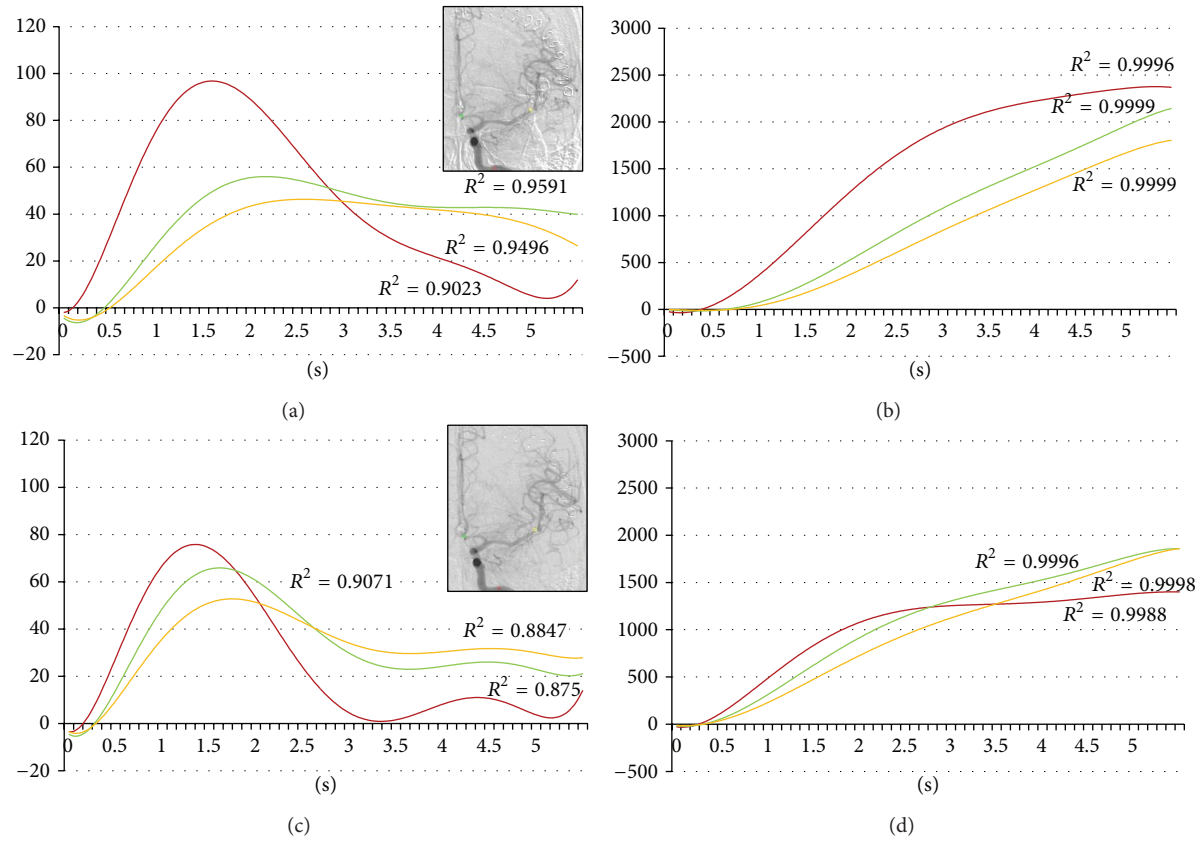

Figure 1: Forty-five-year-old female. Postruptured anterior communicating artery aneurysm clipping. Angiography was done on the ninth day after the subarachnoid hemorrhage. Red, yellow, and green indicate the internal carotid artery, the middle cerebral artery, and the anterior cerebral artery, respectively. (a) Time-density curve for contrast medium for preintra-arterial injection of vasodilator. (b) Integration curve for contrast medium for preintra-arterial injection of vasodilator. (c) Time-density curve for contrast medium for postintra-arterial injection of vasodilator. (d) Integration curve for contrast medium for postintra-arterial injection of vasodilator.

Table 1: Analyzed patients. All cases are female and the Fisher Group of all cases is three.

	Age	Location of aneurysm	Hunt-Hess grade	Day of postclipping DSA	Position of catheter for IA	Onset of symptom after IA	Etiologic artery	GOS at 30 days
Case 1	45	A. com	2	9	Lt. IC	8 hours	Lt. MCA	Good recovery
Case 2	69	Rt. IC	3	7	Rt. IC	2 days	Rt. MCA	Moderate disability
Case 3	69	Lt. MCA	1	9	Lt. IC	1 day	Lt. MCA	Good recovery
Case 4	75	Rt. MCA	2	8	Rt. MCA	1 day	Upper branch of rt. MCA	Moderate disability

A. com: anterior communicating aneurysm. DSA: digital subtraction angiography. GCS: Glasgow Outcome Scale. IA: intra-arterial injection of medicine. IC: internal carotid artery. MCA: middle cerebral artery.

of vasodilator. In this study, we analyze the cerebral blood flow in those cases and discuss an adequate treatment strategy for asymptomatic CVS.

2. Material and Methods

This study was approved by the Hirosaki University Ethics Committee and we acquired written, informed consent for this study from patients and/or family.

From January 2007 to December 2012 we treated 281 cases of SAH within 72 hours after aneurysm rupture. We maintained normovolemia, along with administering intravenous fasudil hydrochloride. Routine postoperative angiography was carried out 9 ± 2 days after the onset of SAH.

During the first year of this study, we undertook 51 IA therapies in 40 patients. Four asymptomatic vasospasm cases are included in this series (Table 1). Pulsatile IA injection of 30 mg fasudil hydrochloride and vasodilators was applied to

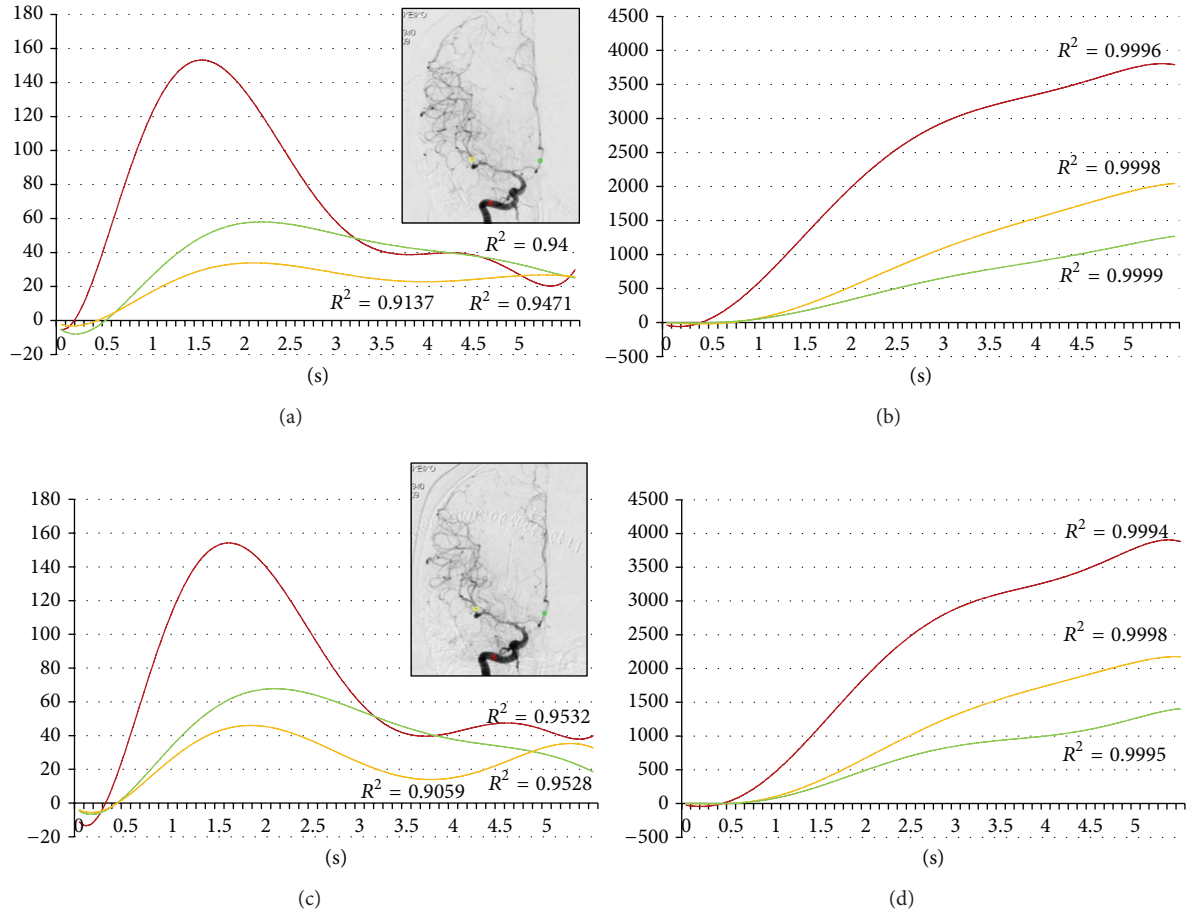

FIGURE 2: Sixty-nine-year-old female. Postruptured right internal carotid artery aneurysm clipping. Angiography was done on the seventh day after the subarachnoid hemorrhage. Red, yellow, and green indicate the internal carotid artery, the middle cerebral artery, and the anterior cerebral artery, respectively. (a) Time-density curve for contrast medium for preintra-arterial injection of vasodilator. (b) Integration curve for contrast medium for the preintra-arterial injection of vasodilator. (c) Time-density curve for contrast medium for the postintra-arterial injection of vasodilator. (d) Integration curve for contrast medium for the postintra-arterial injection of vasodilator.

spastic arteries. All four cases improved vasospasm immediately following intervention (Figures 1, 2, 3, and 4). But all cases changed to symptomatic CVS within 48 hours. We subsequently analyzed the parameters of cerebral blood flow to clarify these unexpected events.

Digital subtraction angiography (DSA) was performed by a certified neurosurgeon. A 4Fr or 6Fr catheter was inserted via the femoral artery into each internal carotid artery, and its tip was set at the level of the second cervical vertebra. Six milliliters of contrast agent was injected into the internal carotid artery at 4 mL/s by autoinjector. Images were obtained at a rate of six frames per second with the use of a DSA unit (Artis dBA Twins, Siemens, Germany) with a pixel matrix of 1024×1024, and the DSA images were stored in the computer system.

The regions of interest (ROI) were set in the vertical petrous portion of the internal carotid artery (red), the etiologic artery (yellow), and the nonetiologic artery (green) on the images of an anteroposterior projection (Figures 1–4) with avoidance of veins. The time-density curve and integration curve of the contrast media in each ROI were obtained using a U11437 luminance analyzer (Hamamatsu Photonics, Shizuoka, Japan) from the series of DSA images. The initial time was defined as the time at which each curve took an upward turn in the ROI of C5, and the entire first-pass curve was established during 6 seconds [25–27]. The time-density curve and the integration curve were fitted to polynomial approximation, and a coefficient of determination (R^2) that was over 0.8 was accepted [28].

The time to peak opacification was defined as time to peak (TTP). The mean transit time (MTT) in each ROI was determined as $\sum(0\text{–}\infty)Ct/\sum(0\text{–}\infty)C$, where C is the quantity of contrast medium remaining at the site and t is the time after the contrast media is injected. The time to half peak of the integration curve was defined as the MTT [29]. Relative blood flow (rBF) was calculated as

$$\frac{\sum(0\text{–}\infty)\,Crt/\{\sum(0\text{–}\infty)\,Crt/\sum(0\text{–}\infty)\,Cr\}}{\{\sum(0\text{–}\infty)\,Ctic/\sum(0\text{–}\infty)\,Cic\}} = \sum(0\text{–}\infty)\,Cr * \sum(0\text{–}\infty)\,Cic/\sum(0\text{–}\infty)\,Ctic, \quad (1)$$

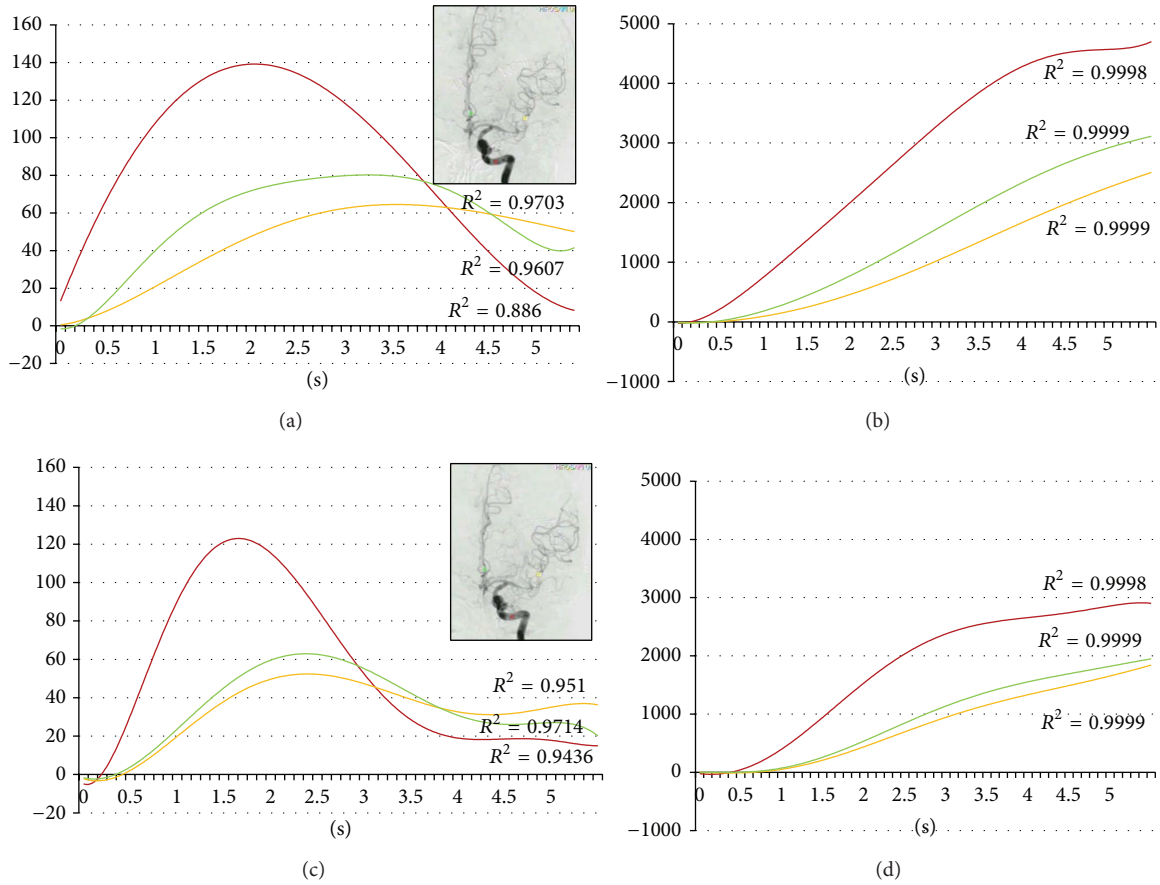

Figure 3: Sixty-nine-year-old female. Postruptured left middle cerebral artery aneurysm clipping. Angiography was done on the ninth day after the subarachnoid hemorrhage. Red, yellow, and green indicate the internal carotid artery, the middle cerebral artery, and the anterior cerebral artery, respectively. (a) Time-density curve for contrast medium for the preintra-arterial injection of vasodilator. (b) Integration curve for contrast medium for the preintra-arterial injection of vasodilator. (c) Time-density curve for contrast medium for the postintra-arterial injection of vasodilator. (d) Integration curve for contrast medium for the postintra-arterial injection of vasodilator.

where "r" is the region of the artery and "ic" is the internal carotid artery [30].

3. Results

The decreases in TTP of the internal carotid artery, nonetiologic artery, and etiologic artery were 0.05 seconds, 0.60 seconds, and 0.73 seconds, respectively (Table 2). The decreases in MTT of the internal carotid artery, nonetiologic artery, and etiologic artery were 0.28 seconds, 0.38 seconds, and 0.55 seconds, respectively (Table 2). But improvements in rBF of the nonetiologic artery and the etiologic artery were 17% and 10%, respectively (Table 2). Improvement of blood flow in the nonetiologic artery was superior to the etiologic artery (Figure 5).

4. Discussion

We first detected iatrogenic blood flow steal after the IA injection of vasodilator for asymptomatic CVS. It was considered possible that vasodilator flowed into the nonspastic arteries and that these nonetiologic arteries then advanced a much greater supply of blood (Figure 5). The insufficiency of blood flow in the asymptomatic spastic artery induced symptomatic CVS after treatment. Limited vasodilatory reserve and dysfunctional autoregulation have been observed in patients with SAH, while arterial vasodilation is nonuniform [31]. Tekle et al. reported that eleven of 41 endovascular-treated symptomatic CVS cases suffered a renewed occurrence of ischemic symptoms in previously asymptomatic arterial distribution [32]. In our series, eight symptomatic cases (22.2%) underwent multiple IA administrations of medicine. We speculate that the same steal phenomenon occurred in those cases of multiple treatment and led to symptomatic vasospasm. Iwabuchi et al. reported that IA injection of fasudil hydrochloride reduced time to peak in the CVS artery dose dependently [26]. In their experience, 13 asymptomatic vasospasm cases did not change to symptomatic vasospasm, but the blood flow of spastic arteries was not analyzed.

Hesselink et al. reported that the integrated area of the time-density curve of DSA depended on flow, vessel size, amount of iodine injected, framing rate, and the kVp [33]. Only the flow of the artery is different in each ROI case. A change in the integrated area of the time-density curve

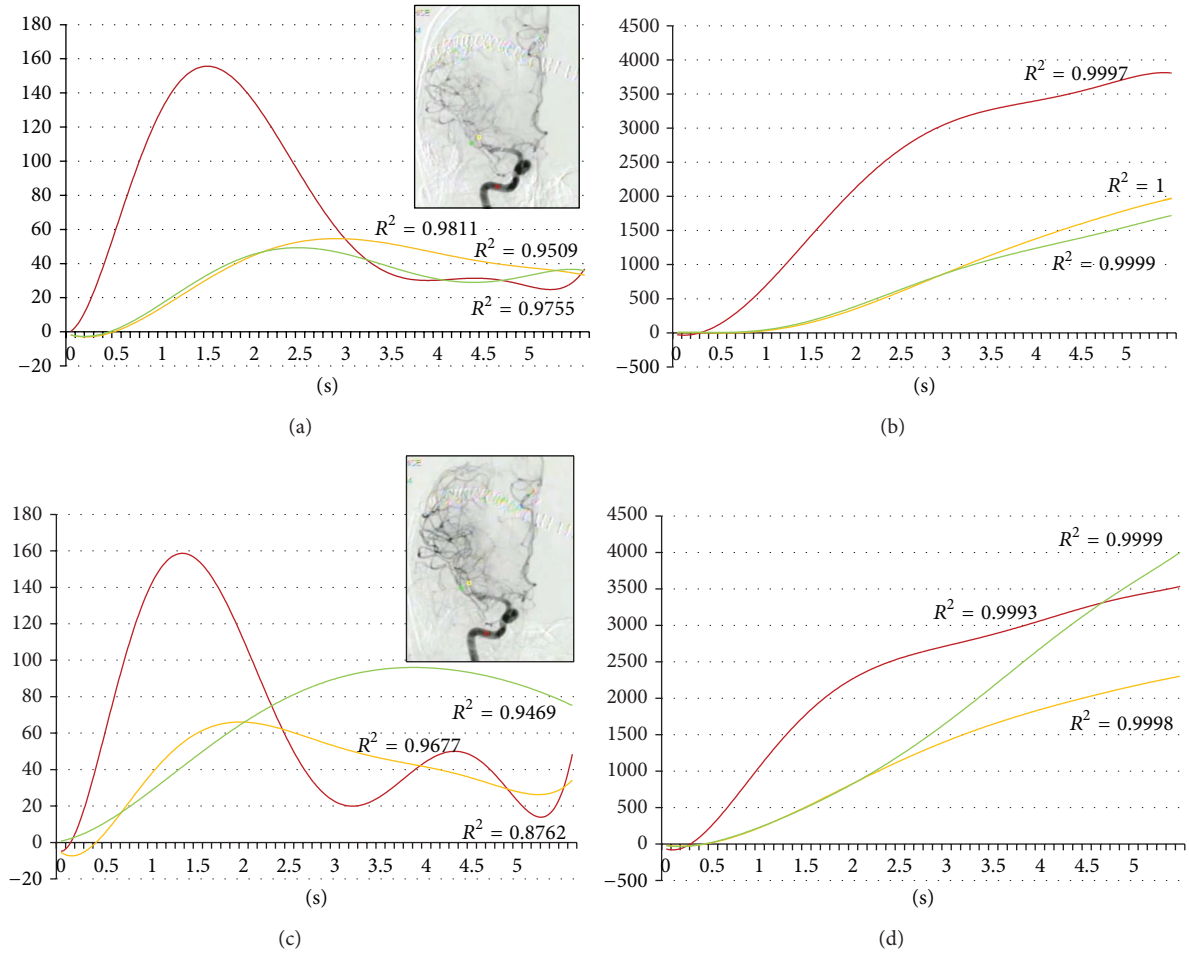

Figure 4: Seventy-five-year-old female. Postruptured right middle cerebral artery aneurysm clipping. Angiography was done on the eighth day after the subarachnoid hemorrhage. Red, yellow, and green indicate the internal carotid artery, the middle cerebral artery, and the anterior cerebral artery, respectively. (a) Time-density curve for contrast medium for the preintra-arterial injection of vasodilator. (b) Integration curve for contrast medium for the preintra-arterial injection of vasodilator. (c) Time-density curve for contrast medium for the postintra-arterial injection of vasodilator. (d) Integration curve for contrast medium for the postintra-arterial injection of vasodilator.

Table 2: Parameters of blood velocity.

	Internal carotid artery	Nonetiologic artery	Etiologic artery
Time to peak (sec)			
Before IA	1.70 ± 0.22	2.65 ± 0.54	2.83 ± 0.61
After IA	1.65 ± 0.31	2.05 ± 0.58	1.9 ± 0.51
Mean transit time (sec)			
Before IA	1.98 ± 0.22	3.08 ± 0.22	3.08 ± 0.15
After IA	1.70 ± 0.37	2.70 ± 0.55	2.53 ± 0.12
Relative blood flow (%)			
Before IA	100	39 ± 13	37 ± 14
After IA	100	56 ± 21	47 ± 18

IA: intra-arterial injection of vasodilator. All values are listed as mean ± standard deviation.

thus means a change in blood flow. Moreover, Lois et al. [30] reported that an inverse integration value for the time-density curve of DSA correlated with blood flow. Calculation of relative blood flow depends on the inverse integration value for the time-density curve for DSA. To calculate the relative blood flow of the spastic artery, we used the integration value of the time-density curve for contrast medium in the internal carotid artery. Kruger et al. reported that the time to maximum opacification can be used to determine absolute and relative blood flow using a phantom model

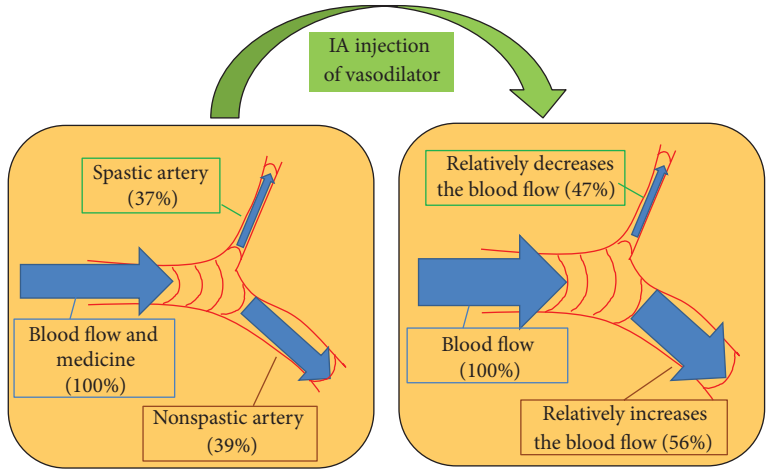

Figure 5: Mechanism of iatrogenic blood steal in a case of asymptomatic vasospasm. Vasodilator easily flows into nonspastic arteries; these nonetiologic arteries then advanced a much greater supply of blood. The improvement in blood flow of the spastic artery is relatively smaller than that of the nonspastic artery (compared to the original).

[34]. Human studies show that blood pressure and heart rate change continually, correlating with cerebral blood flow. To compare pretreatment flow to posttreatment flow we divided the integration value of the time-density curve for contrast medium by the same value for the internal carotid artery, for purposes of standardization.

Even though we performed an IA injection of vasodilator via the M1 portion of the middle cerebral artery in case 4, the patient changed to symptomatic CVS. To prevent a change from asymptomatic CVS to symptomatic CVS, advanced superselective IA injection should be considered. IA injection of vasodilator is carried out directly to the spastic artery via M2, M3, or A2. But this method is difficult and risky for untrained doctors because of the tiny size of the spastic artery. Therefore, prophylactic IA injection of vasodilator for asymptomatic CVS cases is not beneficial.

5. Conclusions

Prophylactic IA injection of vasodilator for asymptomatic CVS cases can produce symptomatic CVS. We do not recommend prophylactic IA of vasodilator for asymptomatic CVS.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of the paper.

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Clinical Study

Impact of Clipping versus Coiling on Postoperative Hemodynamics and Pulmonary Edema after Subarachnoid Hemorrhage

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Volume management is critical for assessment of cerebral vasospasm after aneurysmal subarachnoid hemorrhage (SAH). This multicenter prospective cohort study compared the impact of surgical clipping versus endovascular coiling on postoperative hemodynamics and pulmonary edema in patients with SAH. Hemodynamic parameters were measured for 14 days using a transpulmonary thermodilution system. The study included 202 patients, including 160 who underwent clipping and 42 who underwent coiling. There were no differences in global ejection fraction (GEF), cardiac index, systemic vascular resistance index, or global end-diastolic volume index between the clipping and coiling groups in the early period. However, extravascular lung water index (EVLWI) and pulmonary vascular permeability index (PVPI) were significantly higher in the clipping group in the vasospasm period. Postoperative C-reactive protein (CRP) level was higher in the clipping group and was significantly correlated with postoperative brain natriuretic peptide level. Multivariate analysis found that PVPI and GEF were independently associated with high EVLWI in the early period, suggesting cardiogenic edema, and that CRP and PVPI, but not GEF, were independently associated with high EVLWI in the vasospasm period, suggesting noncardiogenic edema. In conclusion, clipping affects postoperative CRP level and may thereby increase noncardiogenic pulmonary edema in the vasospasm period. His trial is registered with University Hospital Medical Information Network UMIN000003794.

1. Introduction

Cerebral vasospasm is a leading cause of morbidity and mortality in patients with aneurysmal subarachnoid hemorrhage (SAH) and is potentially treatable. However, no definitive treatment for cerebral vasospasm after SAH has been established, and systemic volume management is still critical for the assessment of vasospasm [1–4]. It is therefore important to develop reliable assessment modalities for close monitoring of the hemodynamic status. As transpulmonary thermodilution (PiCCO Plus; Pulsion Medical Systems, Munich, Germany) can measure important hemodynamic parameters without the need for cardiopulmonary catheterization, it has gained increasing acceptance in many intensive care units [5, 6] and for volume management in patients with SAH [7–12].

Since publication of the findings of the International Subarachnoid Aneurysm Trial, endovascular intra-aneurysmal coil embolization has been used more frequently for the treatment of aneurysmal SAH [13–16]. Endovascular coil embolization is thought to be less invasive than aneurysmal clipping, because the duration of the procedure is shorter and there is no need for craniotomy [17]. However, no previous studies have investigated the impact of treatment strategy (clipping versus coiling) on postoperative hemodynamics and pulmonary edema after SAH. This study aimed to use the data prospectively collected by the SAH PiCCO study group to (1) determine whether there are differences in post-

operative hemodynamics and pulmonary edema between patients with SAH who undergo aneurysmal clipping and those who undergo endovascular coiling and (2) investigate the mechanisms underlying postoperative pulmonary edema.

2. Methods

2.1. Study Population. Patients were included if they had a ruptured cerebral aneurysm diagnosed by cerebral angiography or three-dimensional angiography. The exclusion criteria were (1) <15 years of age, (2) absence of brainstem reflexes, (3) pregnancy, and (4) severe cardiopulmonary dysfunction requiring percutaneous cardiopulmonary support. Patients with rebleeding during the postoperative study period were also excluded because the accuracy of diagnosis of delayed cerebral ischemia (DCI) and the degree of pulmonary edema could be affected by rebleeding. The SAH PiCCO study was a multicenter prospective cohort study of SAH patients admitted to the nine participating Japanese university hospitals. The study was approved by the appropriate ethics committees of all participating institutions, and written informed consent for treatment was obtained from all patients or their next of kin. The study was registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (http://apps.who.int/trialsearch/trial.aspx?trialid=JPRN-UMIN000003794): UMIN-CTR ID UMIN000003794.

All patients admitted to the nine participating institutions with aneurysmal SAH between October 2008 and March 2012 were screened for eligibility. Patients who were monitored using the PiCCO system during the perioperative period were included in the study. All patients underwent aneurysm treatment (clipping or coiling) within 48 hours of the onset of symptoms of SAH. Treatment decisions were at the discretion of the attending physician.

2.2. Transpulmonary Thermodilution (PiCCO) Monitoring. All patients were monitored using PiCCO Plus from days 1 to 14 after SAH. A 4-F thermistor-tipped arterial catheter (PV2014L16; Pulsion Medical Systems) was inserted into the femoral or brachial artery. The arterial catheter and a central venous catheter were connected to pressure transducers and to the PiCCO Plus system for monitoring. Global ejection fraction (GEF; normal range 25–35%), cardiac index (CI; 3–5 L/min/m^2), and systemic vascular resistance index (SVRI; 1700–2400 dyn/sec/m^2) were measured to assess cardiac contractility and afterload. Global end-diastolic volume index (GEDI; 680–800 mL/m^2) was measured to assess preload. Extravascular lung water index (EVLWI; 3.0–10.0 mL/kg) and pulmonary vascular permeability index (PVPI; 1.0–3.0) were measured to assess pulmonary parameters. The parameters were determined with continuous cardiac output calibration by triplicate central venous injections of 15 mL of ice-cold saline (<8°C). Cardiac output was calculated by analysis of the thermodilution curve followed by pulse-contour analysis for continuous monitoring. Details of the PiCCO monitoring protocol have been described elsewhere [10, 11, 18].

2.3. Postoperative Management. Perioperative care was performed according to the standardized protocol provided by the current American Heart Association guidelines [1]. Patients underwent brain natriuretic peptide (BNP) quantification on postoperative day 1 and laboratory testing of the blood until day 14. Volume management was monitored throughout the analysis until day 14. Intracranial and cerebrospinal fluid pressure were controlled by ventricular, cisternal, or spinal drainage. Blood transfusion was performed when the hemoglobin and hematocrit levels were below the lower limits of the normal ranges (<10 g/dL hemoglobin, <35% hematocrit). Triple-H (hypervolemia, hypertension, and hemodilution) therapy was administered for symptomatic vasospasm at the discretion of the attending physician. DCI was defined as symptomatic cerebral vasospasm or cerebral infarction caused by cerebral vasospasm. In comatose patients, symptomatic vasospasm was defined as cerebral infarction due to vasospasm observed on angiography. Magnetic resonance imaging, computed tomography angiography, and transcranial Doppler ultrasonography were also used to detect vasospasm in patients with DCI.

2.4. Statistical Analysis. Data are presented as median values with 95% confidence intervals. Data were tested for normality of distribution and equal standard deviations using GraphPad InStat (Version 3.10; GraphPad Software, USA) to determine whether parametric or nonparametric assumptions should be used for each statistical test. Comparisons between groups were performed using the Mann-Whitney test for continuous variables and the χ^2 test for categorical variables. Correlations between C-reactive protein (CRP) level and postoperative brain BNP level were analyzed using the Pearson rank correlation test (which measures the linear relationship between two variables), because we expected a linear relationship. Multivariate regression analysis was performed to identify factors associated with high EVLWI (>10 mL/kg) in the early period (days 1–3) and the vasospasm period (days 6–8) using SPSS (Version 15.0; SPSS Japan Inc., Tokyo, Japan). Odds ratios for high EVLWI were adjusted for age, sex, World Federation of Neurological Surgeons (WFNS) grade, Fisher grade, treatment (clipping or coiling), transfusion, triple-H therapy, CRP level, PVPI, GEF, and GEDI. The text and figure legends describe the statistical tests used. Unless stated otherwise, differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Patient Characteristics. This study enrolled 204 patients. After exclusion of two patients who underwent surgical trapping with bypass, 202 patients were included in the analyses (Table 1). Clipping was performed in 160 patients and coiling was performed in 42 patients. Surgical clipping was preferably performed for middle cerebral artery aneurysms and endovascular coiling was preferably performed for basilar/vertebral artery aneurysms. There were no significant differences in age, Fisher grade, aneurysm size, preoperative rebleeding rate, DCI, or Glasgow Outcome Scale score between the clipping and coiling groups. However, patients in the coiling group had significantly higher WFNS grades than those in the clipping group ($P = 0.02$). Patients

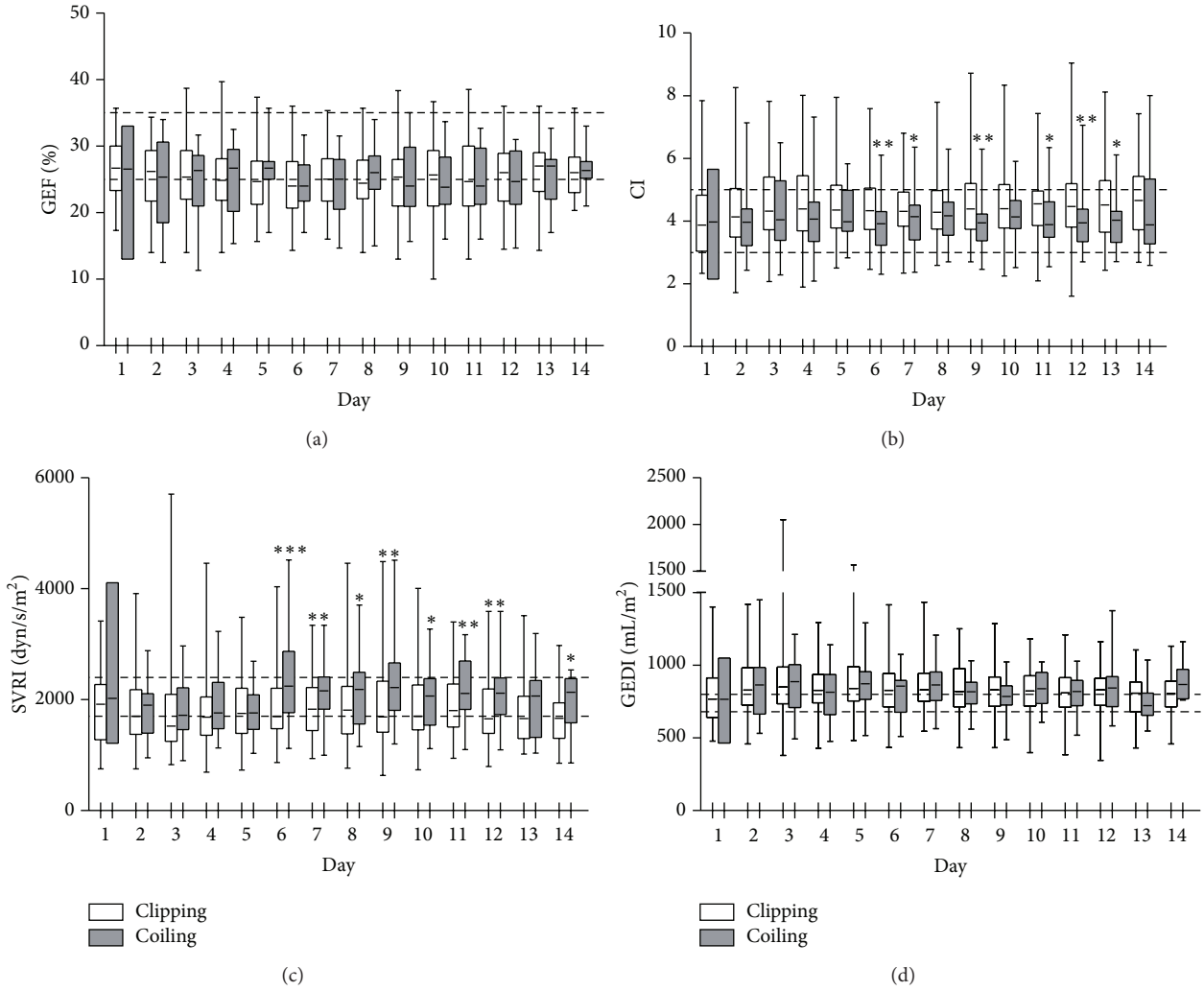

Figure 1: Values for GEF (a), CI (b), SVRI (c), and GEDI (d) for 14 days after SAH. The dotted lines indicate the upper and lower limits of the normal ranges. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Mann-Whitney test. GEF: global ejection fraction; CI: cardiac index; SVRI: systemic vascular resistance index; and GEDI: global end-diastolic volume index.

in the clipping group were more likely to receive transfusion (62.5% versus 31.0%; $P = 0.002$) and triple-H therapy (40.0% versus 14.3%; $P = 0.005$) than those in the coiling group.

3.2. Postoperative Hemodynamic Parameters after Clipping and Coiling. The time courses of postoperative hemodynamic parameters are shown in Figure 1. GEF, a measure of cardiac contractility, was around the lower limit of the normal range throughout the study period and was not significantly different between the clipping and coiling groups (Figure 1(a)). CI and SVRI were measured to assess afterload (Figures 1(b) and 1(c)). In the early period after SAH, CI was within the normal range and SVRI was around the lower limit of the normal range in both groups. In the vasospasm period (from day 6 onwards), CI was significantly lower and SVRI was significantly higher in the coiling group than in the clipping group. GEDI, a measure of preload, was above the upper limit of the normal range in both groups (Figure 1(d)). These results suggest that afterload mismatch may occur even in the vasospasm period after SAH in cases of aggressive volume loading. In this study, afterload mismatch was more likely to occur in the coiling group than in the clipping group, probably because the coiling group had a higher proportion of patients with poor WFNS grade.

3.3. Postoperative Pulmonary Parameters after Clipping and Coiling. The time courses of postoperative pulmonary parameters are shown in Figure 2. Overall, EVLWI was around the upper limit of the normal range throughout the study period in both groups and was significantly higher in the clipping group than in the coiling group on days 5 and 7 ($P = 0.049$ and $P = 0.03$, resp.; Figure 2(a)). Evaluation of EVLWI according to the WFNS grade showed that EVLWI was significantly higher in the clipping group on days 6 and 7 in patients with WFNS grades 1–3 ($P = 0.04$ and $P = 0.04$, resp.) and on days 4, 5, and 9 in patients with WFNS grades 4-5 ($P = 0.04$, $P = 0.03$, and $P = 0.03$, resp.). However, PVPI was within the normal range throughout the study period

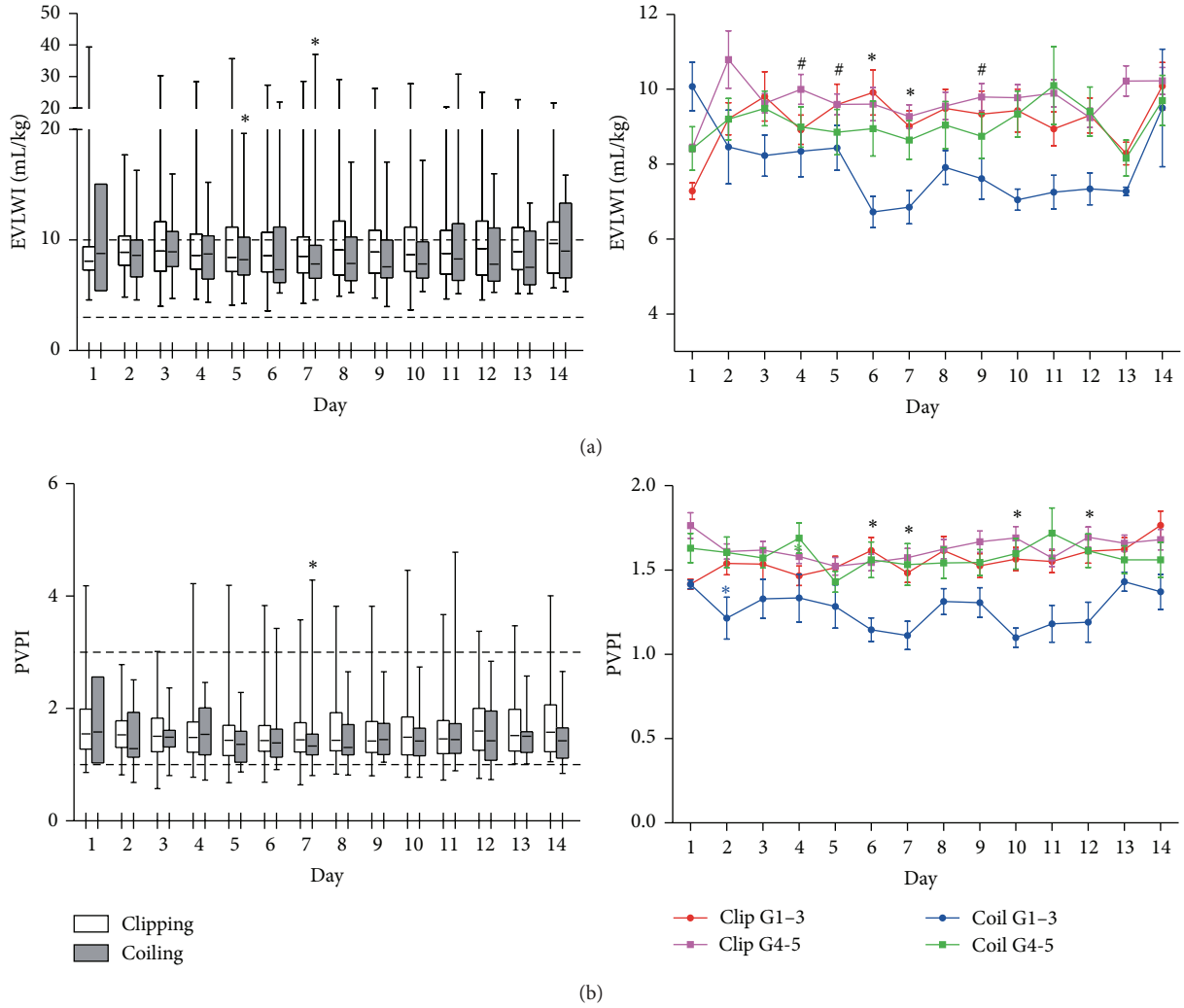

FIGURE 2: Values for EVLWI (a) and PVPI (b) for 14 days after SAH. The dotted lines indicate the upper and lower limits of the normal ranges. The colored lines show EVLWI and PVPI according to the WFNS grade. $^{*}P < 0.05$, comparison between the clipping and coiling groups in WFNS grades 1–3; $^{\#}P < 0.05$, comparison between the clipping and coiling groups in WFNS grades 4-5, Mann-Whitney test. EVLWI: extravascular lung water index and PVPI: pulmonary vascular permeability index.

in both treatment groups and was significantly higher in the clipping group than in the coiling group on day 7 ($P = 0.048$; Figure 2(b)). Evaluation of PVPI according to the WFNS grade showed that PVPI was significantly higher in the clipping group on days 6, 7, 10, and 12 in patients with WFNS grades 1–3 ($P = 0.03$, $P = 0.02$, $P = 0.02$, and $P = 0.03$, resp.). These results suggest that postoperative pulmonary edema was more likely to occur in the clipping group in the vasospasm period with PVPI elevation.

3.4. Associations among CRP Level, Cardiopulmonary Parameters, and Outcome. In both groups, the CRP level increased until day 3 and then decreased gradually (Figure 3(a)). The CRP level was significantly higher in the clipping group than in the coiling group on days 2, 3, 4, 6, and 7 ($P = 0.03$, $P = 0.006$, $P = 0.005$, $P = 0.02$, and $P = 0.04$, resp.). Evaluation of the CRP level according to the WFNS grade showed that there was a moderate-to-weak positive correlation between the CRP level on day 3 and the postoperative BNP level in patients with WFNS grades 4-5 ($r = 0.29$, $P = 0.03$; Figure 3(b)). Evaluation of outcome according to the Glasgow Outcome Scale score at discharge showed that the CRP level on day 3 and EVLWI on day 7 were significantly higher in patients discharged in a vegetative state than in those not discharged in a vegetative state ($P < 0.05$; Figure 4).

Multivariate analysis to identify the risk factors for high EVLWI (>10 mL/kg) showed that PVPI and GEF were independently associated with high EVLWI in the early period, suggesting cardiogenic edema ($P = 0.001$ and $P = 0.004$, resp.; Table 2), and that CRP level and PVPI, but not GEF, were independently associated with high EVLWI in the vasospasm period, suggesting noncardiogenic edema ($P = 0.022$, $P = 0.001$, and $P = 0.507$, resp.; Table 3).

4. Discussion

This is the first study to evaluate differences in postoperative hemodynamics and pulmonary edema between patients who

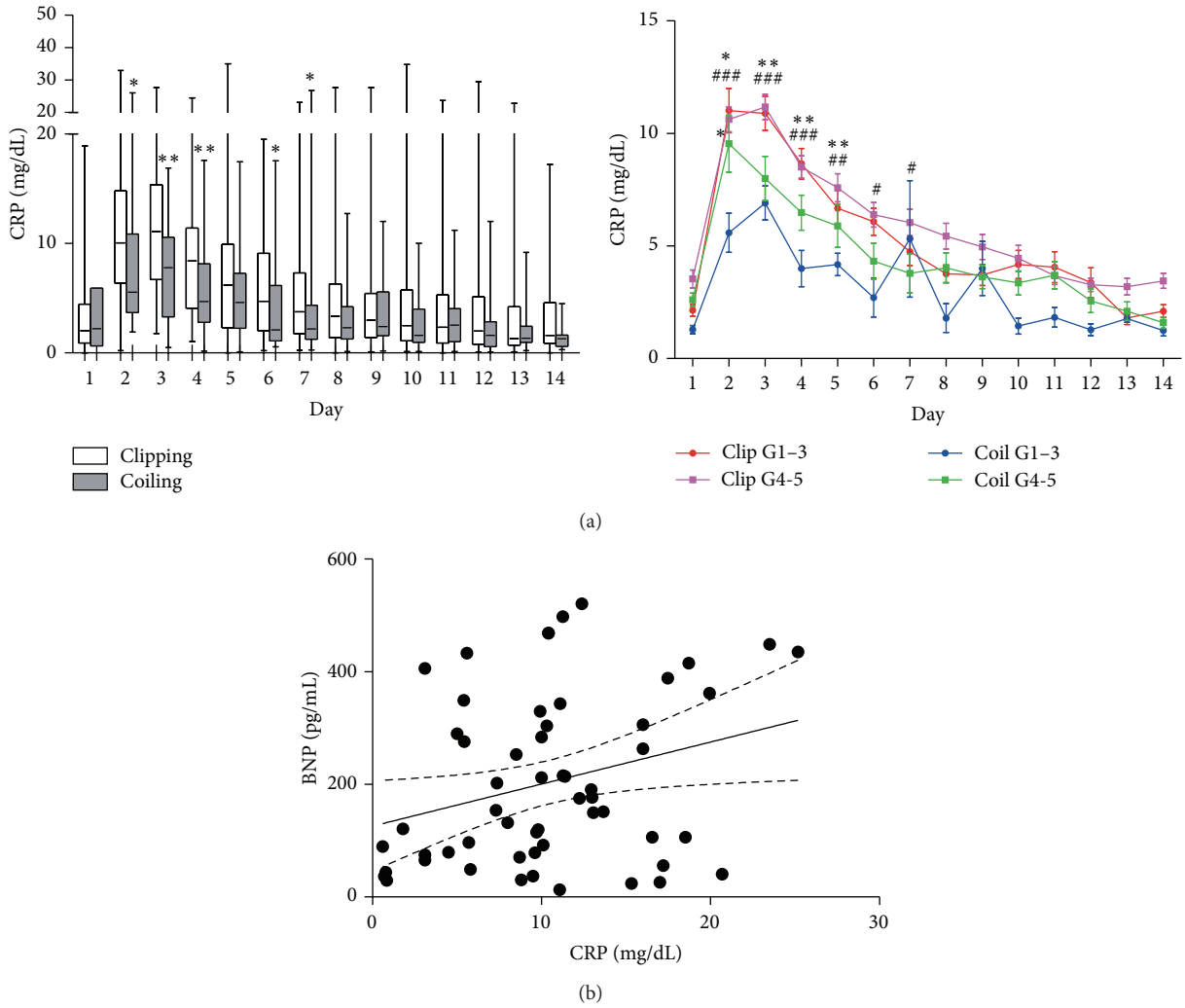

Figure 3: (a) CRP levels for 14 days after SAH. The colored lines show CRP level according to the WFNS grade. $^{*}P < 0.05$, $^{**}P < 0.01$, comparison between the clipping and coiling groups in WFNS grades 1–3; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, comparison between the clipping and coiling groups in WFNS grades 4-5, Mann-Whitney test. (b) Linear regression curves for correlation between the CRP and BNP levels in WFNS grades 4-5. Pearson rank correlation test. CRP: C-reactive protein and BNP: brain natriuretic peptide.

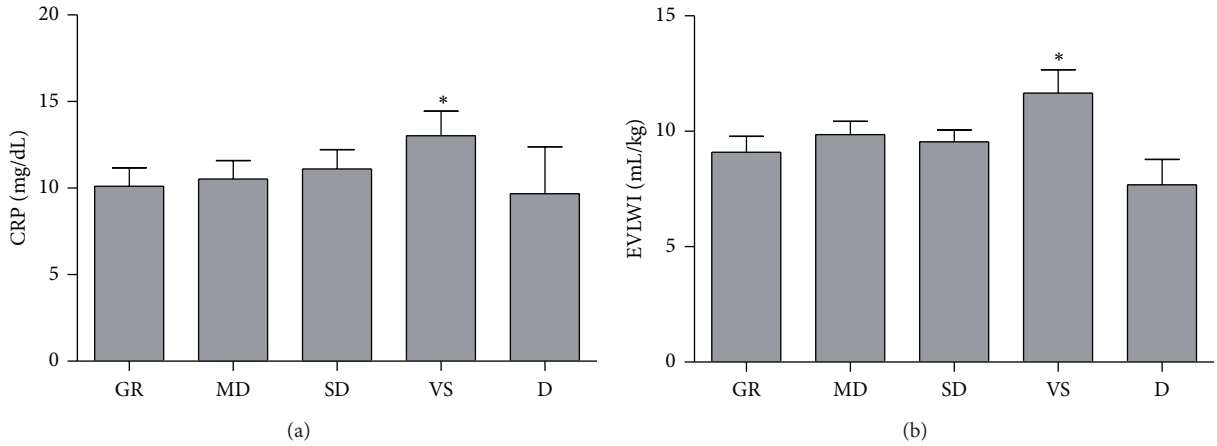

Figure 4: CRP level and EVLWI according to Glasgow Outcome Scale score after SAH. $^{*}P < 0.05$, one-way analysis of variance, Tukey-Kramer multiple comparisons test. GR: good recovery; MD: moderate disability; SD: severe disability; VS: vegetative state; and D: death.

Table 1: Clinical characteristics of patients.

	Clipping (%)	Coiling (%)	*P* value
N	160	42	
Age	63.4 ± 13.1	61.9 ± 11.8	n.s
Male sex	49(30.6)	16(38.1)	n.s
WFNS grade			0.02
I	17(10.6)	3(7.1)	
II	30(18.8)	5(11.9)	
III	11(6.9)	1(2.4)	
IV	43(26.9)	8(19.0)	
V	59(36.9)	25(59.5)	
Fisher grade			n.s
1	1(0.6)	0(0.0)	
2	9(5.6)	2(4.8)	
3	92(57.5)	26(61.9)	
4	58(36.3)	14(33.3)	
Aneurysm size	6.6 ± 5.5	7.1 ± 4.1	n.s
Aneurysm location			
ACA	47(29.4)	11(26.2)	n.s
MCA	61(38.1)	0(0)	<0.0001
ICA	46(28.8)	9(21.4)	n.s
BA/VA	6(3.4)	22(52.4)	<0.0001
Rebleeding	3(1.8)	1(2.3)	n.s
Transfusion	100(62.5)	13(31.0)	0.002
Triple-H therapy	64(40.0)	6(14.3)	0.005
DCI	36(22.5)	10(23.8)	n.s
GOS			n.s
GR	38(23.8)	8(19.0)	
MD	43(26.9)	8(19.0)	
SD	45(28.1)	14(33.3)	
VS	25(15.0)	9(21.4)	
D	9(5.6)	3(7.1)	

WFNS: World Federation of Neurological Surgeons; DCI: delayed cerebral ischemia; GOS: Glasgow Outcome Scale; GR: good recovery; MD: moderate disability; SD: severe disability; VS: vegetative state; D: death; ACA: anterior communicating artery; MCA: middle cerebral artery; ICA: internal carotid artery; and BA/VA: basilar artery/vertebral artery; n.s: not significant.

Table 2: ORs for high EVLWI (>10 mL/kg) in the early period.

Variable	Adjusted OR	95% CI	*P* value
Age	0.99	0.94–1.05	0.842
Sex female	2.85	0.43–19.10	0.280
WFNS grade	1.27	0.68–2.38	0.457
CT Fisher grade	0.56	0.19–1.64	0.292
Clipping	1.31	0.29–5.84	0.726
Transfusion	1.95	0.28–13.53	0.500
CRP day 3	0.97	0.85–1.12	0.687
PVPI day 3	27.54	3.59–211.42	0.001
GEF day 3	0.75	0.62–0.91	0.004

WFNS: World Federation of Neurological Surgeons; CRP: C-reactive protein; PVPI: pulmonary vascular permeability index; and GEF: global ejection fraction.

underwent surgical clipping and endovascular coiling for aneurysmal SAH. Based on analysis of the PiCCO SAH group database, we present the following findings. First, postoperative hemodynamics are similar in both groups in the early period, and afterload mismatch may occur in the vasospasm period with aggressive volume loading. Previously published reports also suggest that hypervolemia can be harmful in patients with SAH [19, 20]. In this prospective cohort study, patients with severe vasospasm received triple-H therapy combined with precise monitoring of cardiopulmonary function using PiCCO, and prophylactic hypervolemia was induced in selected cases at the discretion of the attending physician. The results of a North American practice survey also showed that physicians were still inducing prophylactic hypervolemia in selected patients with severe vasospasm [21]. The possibility of cardiopulmonary dysfunction due to aggressive hypervolemia should be considered in these patients. Afterload mismatch occurred more frequently in the coiling group, probably because this group had a higher proportion of patients with poor WFNS grade. Second, postoperative pulmonary edema occurred more frequently in the clipping group in the vasospasm period with PVPI elevation. Finally, a high postoperative CRP level may be a risk factor for noncardiogenic pulmonary edema in the vasospasm period.

Table 3: ORs for high EVLWI (>10 mL/kg) in the vasospasm period.

Variable	Adjusted OR	95% CI	*P* value
Age	1.04	0.99–1.09	0.131
Sex female	2.77	0.57–13.4	0.206
WFNS grade	1.15	0.67–1.98	0.614
CT Fisher grade	0.37	0.12–1.22	0.103
Clipping	1.19	0.26–5.54	0.821
Transfusion	1.53	0.29–8.13	0.621
Triple H	0.80	0.23–2.83	0.729
CRP day 3	1.15	1.02–1.29	0.022
PVPI day 7	35.97	4.27–303	0.001
GEF day 7	1.05	0.91–1.22	0.507

WFNS: World Federation of Neurological Surgeons; CRP: C-reactive protein; PVPI: pulmonary vascular permeability index; and GEF: global ejection fraction.

It is sometimes difficult to evaluate pulmonary edema on X-ray, whereas PiCCO is a useful modality for quantitative evaluation of the status of the lungs [5, 6]. EVLWI was around the upper limit of the normal range throughout the study period, suggesting postoperative accumulation of extravascular lung water even without any abnormal findings on X-ray. Pulmonary edema was detected on X-ray in 19.8% of the patients in this study. The true incidence of pulmonary complications after SAH remains unclear because of the lack of quantitative hemodynamic measurements to date.

The International Subarachnoid Aneurysm Trial reported lower rates of death and disability after endovascular treatment of ruptured intracranial aneurysms than after neurosurgical treatment [13–16]. Based on these results, the treatment of patients with ruptured intracranial aneurysms has changed significantly over recent years. In many centers, coiling has become the treatment modality of choice when both coiling and clipping are considered suitable in a particular patient. However, very few reports have shown differences between clipping and coiling in terms of invasiveness [17, 22]. As the brain is not manipulated during endovascular coiling,

the risk of brain damage may be reduced compared with clipping. Moreover, the time of operation under anesthesia is much shorter for coiling than for clipping. These differences could affect the catecholamine surge during the acute stage after SAH, thereby affecting postoperative cardiopulmonary function [23, 24], which is very important for the volume management of vasospasm after SAH.

Neurogenic pulmonary edema is a clinical syndrome characterized by acute onset of pulmonary edema following a significant central nervous system insult such as SAH, spinal cord injury, traumatic brain injury, or intracranial hemorrhage [25]. Neurogenic pulmonary edema is thought to be caused by a catecholamine surge that results in cardiopulmonary dysfunction [26], and several mechanisms have been proposed: neurocardiac [27], neurohemodynamic [28], blast theory [29], and pulmonary venule adrenergic hypersensitivity [30]. Our data show that EVLWI was initially high in both groups, probably because of a catecholamine surge after the onset of SAH. However, the time course of EVLWI was different between patients who underwent clipping and coiling. EVLWI stayed high in the clipping group but gradually improved in the coiling group. In this study, we focused on CRP level because the postoperative CRP level was significantly higher in the clipping group than in the coiling group. Recently, a high postoperative CRP level has been reported to be a predictive factor for poor outcome after SAH, although the mechanism underlying this association is still undetermined [31, 32]. In this study, we found two types of pulmonary edema after SAH: cardiogenic pulmonary edema due to poor cardiac contractility in the early period and noncardiac pulmonary edema due to elevation of the postoperative CRP level, which mainly occurred after clipping. This is consistent with previously reported findings [10]. The significant correlation between the CRP level (which increases for 24–48 hours after an inflammatory insult) and the postoperative BNP level strongly supports our hypothesis that the postoperative CRP level affects cardiopulmonary function after SAH. Although there is no established evidence of associations among CRP level, EVLWI, and BNP, we consider that systemic inflammatory response syndrome in response to the brain injury after SAH [33–35] may contribute to the cardiopulmonary complications. Further hemodynamic data are needed to clarify the underlying mechanisms.

BNP is a hormone primarily produced in the left cardiac ventricle in response to cardiac wall stretch, which causes diuresis through direct natriuretic action, increased cardiac output, or decreased aldosterone level [36]. In a small series of SAH patients, plasma concentrations of BNP were found to be elevated [37–39], but the reasons for this are still unclear. Our findings indicate that the increased postoperative CRP level could result in increased elevation of the BNP level, via increased EVLWI due to cardiogenic or noncardiogenic pulmonary edema. Takotsubo cardiomyopathy, which is associated with the catecholamine surge after SAH [40], may also play a role in the cardiac insufficiency. Finally, we found that the postoperative CRP level in the early period and EVLWI in the vasospasm period were significantly higher in patients with a vegetative state after SAH than in those not in a vegetative state. This indicates that postoperative CRP level can predict pulmonary complications that affect morbidity after SAH.

Taken together, careful monitoring of the hemodynamic and pulmonary parameters until the end of the vasospasm period is mandatory to avoid development of cardiac insufficiency or pulmonary complications, which could contribute to the outcome after SAH.

Several limitations of this study should be discussed. First, catecholamine levels were not collected in the PiCCO study. The BNP level was recorded once, within 3 days after SAH (postoperative day 1), and the time course of the BNP level was therefore not available. Second, cardiac function was evaluated using only PiCCO data, and regular echocardiography or electrocardiography evaluations were not performed. Nevertheless, bedside monitoring using PiCCO is a powerful, established tool for the assessment of cardiopulmonary hemodynamics [5–11]. Third, distribution of the aneurysm location was different between patients who undergo clipping and coiling, which could affect cardiopulmonary function [41, 42]. Impact of aneurysm location on cardiopulmonary dysfunction could be clarified by another substudy. Fourth, sedation and mechanical ventilation data were not recorded in the PiCCO study, and parameters affected by mechanical ventilation were therefore not analyzed in this study. It has previously been reported that EVLWI is not affected by mechanical ventilation [43]. Finally, intracranial pressure monitoring was not performed or recorded in this study which might affect EVLWI [41].

In conclusion, this is the first study to evaluate the differences in postoperative hemodynamics and pulmonary parameters between clipping and coiling for aneurysmal SAH. Postoperative hemodynamics were similar in both groups in the early period, and afterload mismatch sometimes occurred in the vasospasm period. Clipping may increase noncardiogenic pulmonary edema in the vasospasm period, resulting from the increased postoperative CRP level. The postoperative CRP level may predict pulmonary complications after SAH.

Abbreviations

SAH: Subarachnoid hemorrhage
DCI: Delayed cerebral ischemia
GEF: Global ejection fraction
CI: Cardiac index
SVRI: Systemic vascular resistance index
GEDI: Global end-diastolic volume index
EVLWI: Extravascular lung water index
PVPI: Pulmonary vascular permeability index
CRP: C-reactive protein
BNP: Brain natriuretic peptide
WFSN: World Federation of Neurological Surgeons.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Nobutaka Horie and Mitsutoshi Iwaasa contributed equally to this work.

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Research Article

Magnesium Lithospermate B, an Active Extract of *Salvia miltiorrhiza*, Mediates sGC/cGMP/PKG Translocation in Experimental Vasospasm

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Background. Soluble guanylyl cyclases (sGCs) and Ras homolog gene family, member A (rhoA)/Ras homolog gene family kinase(rho-kinase) plays a role in vascular smooth muscle relaxation in subarachnoid hemorrhage (SAH). It is of interest to examine the effect of MLB on rhoA/ROCK and sGC/cGMP/PKG expression. *Methods*. A rodent SAH model was employed. Tissue samples were for sGCα1, sGCβ1, PKG, rhoA, ROCK (Western blot), and cGMP (ELISA) measurement. *Results*. MLB morphologically improved convolution of the internal elastic lamina, distortion of endothelial wall, and necrosis of the smooth muscle in the SAH rats. Expressed cGMP, sGCα1, sGCβ1, and PKG in the SAH groups were reduced ($P < 0.01$), and MLB precondition significantly induced cGMP, sGCα1, sGCβ1, and PKG. L-NAME reversed the vasodilation effect of MLB, reduced the bioexpression of PKG and cGMP ($P < 0.01$), and tends to reduce sGCα1 level and induce rhoA, ROCK level in MLB precondition + SAH groups. *Conclusion*. These results demonstrate that sGC/cGMP/PKG and NO/ET pathways play pivotal roles in SAH-induced vasospasm. Through activating sGC/cGMP/PKG pathway and partially by inactivating rho-kinase in a NO-dependent mechanism, MLB shows promise to be an effective strategy for the treatment of this disease entity.

1. Introduction

Subarachnoid hemorrhage (SAH) induced cerebral vasospasm becomes an important subcategory of stroke owing to its high mortality and morbidity, which included delayed neurological deficit, and cardiopulmonary abnormality [1, 2]. Till now, the precise mechanism of this disease contour remains unclear. Several molecular and cellular researches implicate two key hypotheses to cerebral vasospasm: one center on the roles of nitric oxide [3] and endothelins [4] and another converges on the role of ATP-AMP and rhoA/ROCK II [5]. In the study of Carter et al., [3] NO donors were able to relax vascular smooth muscle in the episode of vasospasm. Through mediated on a large conductance Ca^{2+}-dependent K^+ channels, cGMP-dependent protein kinase (PKG) phophorylates and rhoA, NO may attenuates vascular smooth muscle sensitivity to calcium ions [6]. In addition, increased evidence suggests that NO counteracts with endothelins as a vascular modulator in part by mediating the activation of RhoA-ROCK and MLC phosphatase [3, 4]. The study of eNOS knockout mice reticence to diabetic nephropathy also provides some insight into the modulation of soluble guanylyl cyclase (sGC) on NO signaling pathway [7].

The involvement of NO/ET-1 and rhoA/ROCK in SAH induced vasomediated pathways is not completely understood. It has been shown that NO (catalyzed by eNOs, nNos, and iNOs), an endothelium-derived relaxing factor (EDRF), augments with endothelin-1 and inhibits activation of rhoA/ROCK in rat thoracic aorta [8]. ET-1 potentiates the constriction of cerebrovascular smooth muscles induced by oxyhemoglobin through PKC and rhoA/rho kinase pathways

[4, 7]. This is compatible with the findings that a PKC inhibitor, staurosporine, can abolish ET-1 induced contraction in rabbit basilar artery [9]. Moreover, lines of evidence accumulated to present have suggested that activated PKC plays a role in SAH induced vasospasm [10]. For example, phorbol 12, 13-dibutyrate, a PKC activator, can induce a potent and long-lasting contraction of the rabbit basilar artery [11]. In a canine double-hemorrhage model, the membrane translocation of PKCδ from cytosol in the basilar artery was observed after the second injection of autologous blood on the 4th day when vasospasm was robust [10, 12]. Furthermore, intrathecal injection of the PKCδ inhibitor rottlerin inhibited hemorrhage induced vasospastic response as well as the translocation of PKCδ [10].

On the contrary, the key event for triggering vasomotor effect of NO/ET-1 in SAH on the rho-kinase pathway has attracted less attention despite of documented involvement of rho-kinase in cerebral vasospasm following SAH. Rho, a family of small G-proteins, is composed of 3 members: rhoA, rhoB, and rhoC, which behaves like a substantial role in intracellular signaling [8, 13]. Inactive rho is resided as a GDP-bound form in the cytosol. If the vascular smooth muscle cells were stimulated by vasoactive agents and subsequent GDP-GTP exchange, rho may become activated and then translocated to the cell membrane where it interacts with its downstream effectors, rho-kinase (ROCK) [13, 14]. The ROCK family includes two isoforms, namely, ROCK-I and ROCK-II. By phosphorylating myosin light chain phosphatase (MLCP) and inhibiting the activity of phosphatase at the myosin-binding subunit, rho-kinase is stimulated and promotes smooth muscle contraction [15, 16]. In the study of a canine two-hemorrhage model, topical application of a ROCK inhibitor, Y-27632, dose-dependently decreased the spastic response, Rho-kinase activity, and phosphorylation of MLCP in the basilar artery [17, 18]. Moreover, intra-arterial administration of fasudil, another kind of ROCK inhibitor, is also proved to be effective for the treatment of vasospasm in SAH patients [19].

Besides ET-1, nitric oxide (NO), composed of endothelial NOs(eNOs), neuronal NOs(nNOs), and inducible NOs (iNOs), is believed to be an important regulator of the cerebral vascular tone [7, 20]. Upon synthesis, NO binding activates soluble guanylyl cyclase (sGC), a heterodimeric enzyme consisting of α (α_1, α_2, and α_3) and β (β_1, β_2, and β_3) subunits [21]. Activation of sGC leads to convert GTP to cGMP, which in turn activates cGMP-dependent protein kinase (PKG) and cGMP gated ion channel among target cells. In blood vessels, PKG can phosphorylate the inositol, 1, 4, 5-triphosphate receptor and decrease Ca^{2+} concentration and ultimately result in smooth muscle relaxation [9, 22, 23]. Under normal physiological conditions, the productions of ET-1 and NO yield a balanced cerebral vascular tone. However, enhanced generation of ET-1 along with impaired NO production (NO-dependent) or with excessively induced NO (NO-independent) was noted in human and animals with SAH [3]. Nevertheless, the effect of NO/ET-1 production on the sGC/cGMP signaling pathway has not been fully investigated.

In the present study, we aimed to assess the neurological deficits and plasma NO/ET-1 levels, as well as the expressions of PKCδ, rhoA, ROCK-II, and sGC/cGMP/PKG in the basilar artery of rats subject to SAH. The effect of magnesium lithospermate B (MLB) on the NOS/sGC/cGMP/PKG and rhoA/ROCK signaling pathway in SAH was investigated.

2. Materials and Methods

2.1. Materials. Anti-rabbit sGCα1 and sGCβ1 and anti-mouse β-actin antibodies were bought from Sigma-Aldrich (Shanghai, PRC). Anti-rabbit PKG, anti-mouse PKCδ, anti-rabbit ROCK-II, anti-mouse rhoA, and horseradish peroxidase-labeled goat anti-mouse IgG antibodies were obtained from Abcam (Cambridge, MA, USA), BD Transduction Lab (BD Biosciences, California, USA), Upstate Biotech (NY, USA), Santa Cruz Biotech (Santa Cruz Biotechnology, Inc., California, USA), and Chemicon International (CA, USA), respectively. CNM protein extraction kits were from Biochain (Hayward, CA, USA). ET-1 and cGMP ELISA kits were products of Assay Designs (Enzo Life Sciences Inc., NY, USA) and Cayman Chemical (Michigan, USA), respectively. Magnesium Lithospermate B was purified by Ms. Wu SC (Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, ROC), according to the modified protocol described by Tanaka et al. Dried *Salvia miltiorrhiza* were obtained, inoculation with methanol for 8 hr, and then concentrated. MLB was extracted by the aid of a Sephadex LH-20 multiple column chromatography for four times. (Pharmacia Fine Chemicals, Piscataway, NJ, USA).

2.2. Animal Protocols. All the experimental protocols were approved by the Kaohsiung Medical University Hospital animal research committee. Forty-five male Sprague-Dawley rats (BioLasco Taiwan Co., Ltd., authorized by Charles River Lab) weighing 250–350 g were assigned into five groups (9 animals each): Group 1 was designated as sham-operated, Group 2: rats subjected to SAH only, Group 3: SAH rats treated with vehicle (0.1 mol/L PBS), and Groups 4 and 5, SAH animals received 10 mg/kg/day MLB treatment at 24 hr (prevention protocol) and 1 hr (reversal protocol) after SAH, respectively. To induce SAH, rats were anesthetized with a mixture of ketamine (40 mg/kg) and xylazine (6 mg/kg) intraperitoneally (i.p.), and 0.3 mL fresh blood was drawn from the central tail artery and injected into the craniocervical junction using a stereotactic technique. Further nine animals were enrolled in the 0.1 μL NG-nitro-L-arginine methyl ester (L-NAME) intrathecal injection plus 10 mg/kg/day MLB preconditioning SAH rats.

2.3. Hemodynamic Measurements. Heart rate, blood pressure, and rectal temperature were monitored before and after MLB treatment as well as at 48 hr after the induction of SAH by a tail-cuff method (SC1000 Single Channel System, Hatteras Instruments, North Carolina, USA) and rectal thermometer (BIO-BRET-2-ISO, FL, USA).

2.4. Neurological Assessment. Behavior assessment, adapted a modified limb-placing tests (MLPT) [2], was performed

Table 1: Modified limb-placing test (MLPT).

Treatment	Group		
	Ambulation	Placing/stepping reflex	MDI
Normal	0	0	0
SAH	1.28 ± 0.2	1.6 ± 0.16	2.88 ± 0.32
SAH + vehicle	1.22 ± 0.13	1.48 ± 0.22	2.7 ± 0.35
SAH + 1 mg/kg MLB			
Prevention	0.83 ± 0.16	0.78 ± 0.18*	1.61 ± 0.34*
Treatment	0.82 ± 0.15	0.84 ± 0.13	1.66 ± 0.28*
L-NAME + prevention	1.32 ± 0.23	1.45 ± 0.16	2.77 ± 0.39

Results are expressed as the mean ± SEM, $n = 9$; $^*P < 0.01$ versus SAH condition by Mann-Whitney test.

before and at 48 hr after the induction of SAH. Motor function was assessed by two individuals who are blind to the treatment status set, which is composed of two limb-placing tasks of assessment of the sensorimotor assimilation of the forelimb and hindlimb as well as monitoring its response to tactile and proprioceptive stimuli. The summations of neurological examination are scored as normal performance (0 point), incomplete performance (1 point), and no performance (2 points), which were shown in Table 1.

2.5. Tissue Embedding. Basilar artery was collected and the middle third of each artery was left for further analysis. The arterial segment was rinsed in a 0.1 mol/L PBS (pH 7.4) repeatedly, set in 1% osmium tetroxide in PBS at room temperature for 1 hr, and then cleaned with PBS. The vessel segments were dehydrated and immersed in a 1 : 1 mixture of propylene oxide and epoxy resin overnight. The specimens were embedded in 100% epoxy resin and let to polymerize at 60°C for 48 hr the next day. The BAs were cross-sectioned at a thickness of 0.5 μm on an Ultracut E ultramicrotome (Reichert-Jung Ultracut E, Leica, Wetzlar, Germany), launched on glass slides, and stained with 0.5% toluidine blue for morphometric analysis later. The rest of tissue was preserved in liquid N_2 at −70°C until the measurements of protein expression and cGMP levels.

2.6. Basilar Artery Morphometric Studies. Five selected BA cross sections of each animal were analyzed by two investigators blinded to the set groups. The cross-sectional area was automatically measured using computer-assisted morphometry (Image-1/Metamorph Imaging System; Universal Imaging Corp.). The average of five cross sections from a given animal was collected as a single value for this animal. Group data are expressed as the means ± standard error of the means.

2.7. Determination of Endothelial Nitric Oxide Synthase (eNOS). An isolated BA was quantified by adapting a commercial kit to check NOS (Bioxytech NOS Assay Kit, Oxis International, Inc., Portland, OR, USA). Briefly, protein extracted from microvessels was incubated with radiolabeled L-arginine in the presence or in the absence of 1 mmol/L NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, Shanghai, PRC). The reaction was finished by the addition of 50 mmol/L HEPES buffer containing 5 mmol/L EDTA. Radiolabeled L-citrulline was counted after removal of excess L-arginine with an equilibrated resin and then centrifuged.

2.8. Western Blot to Evaluate RhoA, ROCK-II, sGCα1, sGCβ1, and PKCδ Protein Expression. Basilar arteries were homogenized for extraction of cytoplasm (buffer C), nuclear (buffer N), and membrane- (buffer M-) bound proteins, as indicated by the instructions of the manufacturer. Expressions of PKCδ, rhoA, ROCK-II, sGCα1, sGCβ1, and PKG were determined by Western blots using the Bio-Rad protein assay kit (Bio-Rad Lab. Shanghai Co., Ltd., Pudong, Shanghai, PRC) following the manufacturer's instruction. Sampling of equal amount (20 μg/lane) was seceded by a 10% polyacrylamide gel and conveyed to a polyvinylidene difluoride membrane (PerkinElmer Informatics, Cambridge, MA, USA). A vague binding was blocked with a TBST buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) mixed with 5% fat free milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-ROCK (1 : 1000), rabbit anti-sGCα1 (1 : 1000), rabbit anti-sGCβ1 (1 : 1000), mouse anti-PKC (1 : 1000), and mouse anti-β-actin (1 : 15000). The membranes were then washed six times per 5 min with TBST buffer. Appropriate dilution of secondary antibodies (1 : 1000) was incubated for 1 hr. After washing with TBST for six times, the protein bands were detected with the Signal Fire ECL reagent from Cell Signaling Technology (CST) (Cell Signaling Technology, Inc., MA, USA).

2.9. ELISA for Tissue cGMP Levels. Arterial blood was collected in heparin-containing tubes prior to the perfusion. Plasma samples were frozen at −70°C until use. The level of cGMP in the homogenate of BA was measured using an ELISA kit according to the instruction of the manufacturer.

2.10. Statistical Analyses. Group data are expressed as the means ± SEM. Comparison of the neurological deficit scores between groups was performed by the Mann-Whitney test. Comparison of protein expression and biomarkers between groups was done using one-way ANOVA followed by Dunnett's test. Differences were considered significant at a $P < 0.01$.

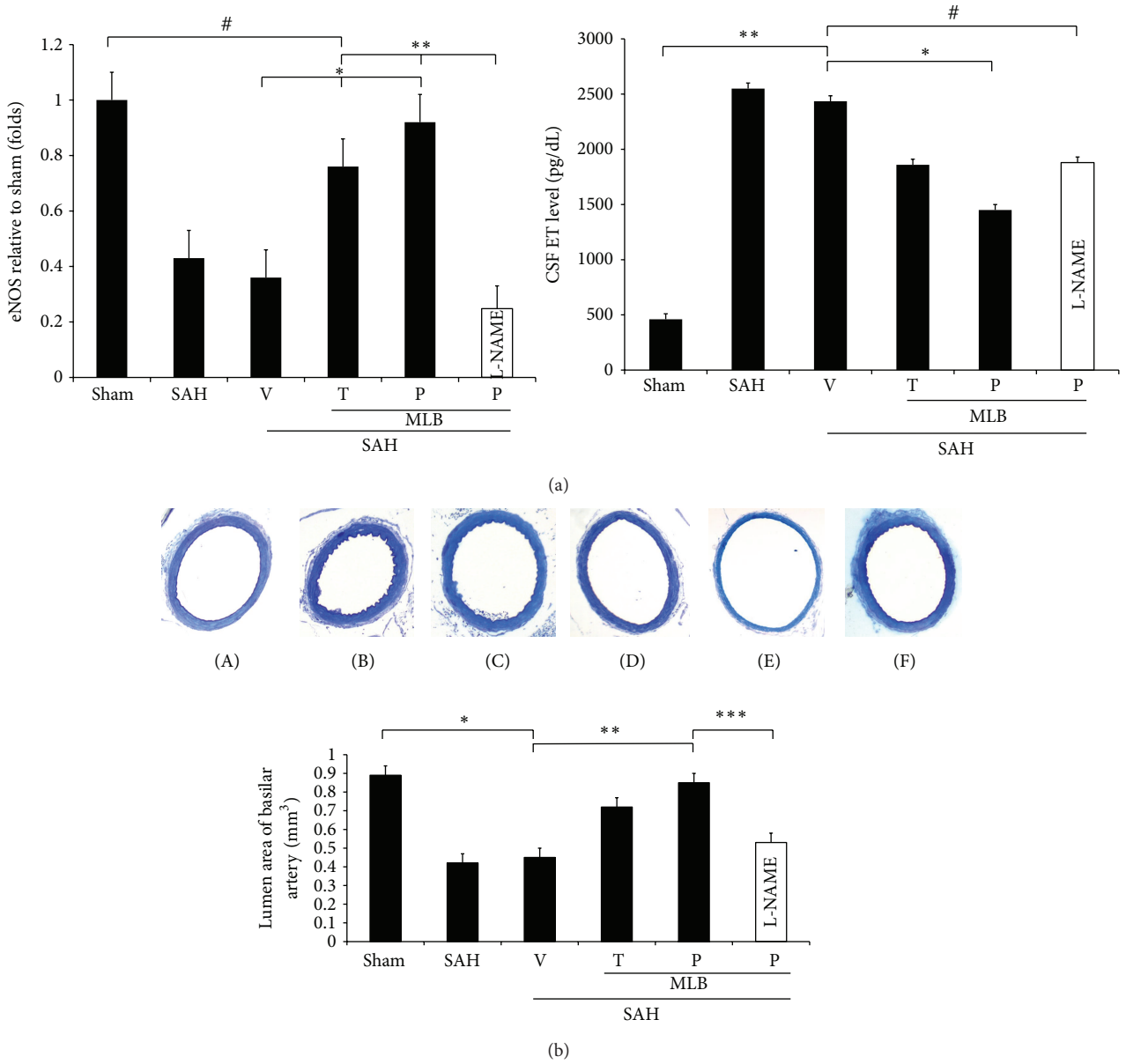

Figure 1: Effect of magnesium lithospermate B (MLB) on endothelial NOS levels (a) and cross section of BAs (b). 10 mg/kg MLB was administered at 1 hr (prevention protocol, P) and 24 hr (treatment protocol, R) after animals were subjected to SAH. BAs eNOS (rt-PCR) was measured. Bottom panels represent micrographs of the cross section of BAs obtained from the healthy controls (A), the SAH only rats (B), the vehicle-treated SAH rats (C), SAH rats received 10 mg/kg/day MLB treatment (D), SAH rats received 10 mg/kg/day, MLB preventive treatment (E), and SAH rats received both 10 mg/kg/day magnesium lithospermate B pretreatment and 1 μg L-NAME (F). Standard bar = 200 μm. $^{*}P < 0.01$: compared with the vehicle + SAH and MLB prevention group. $^{**}P < 0.01$, comparison between sham and the vehicle + SAH groups. $^{***}P < 0.01$: MLB prevention group compared with the L-NAME treatment group. $^{\#}P > 0.05$: compared MLB treatment group with the sham group. Data are mean ± SEM (n = 9/group).

3. Results

3.1. General Observations. By the end of the experiment, there were no statistically significant differences among the control and experimental groups in the following physiological parameters evaluated, which enrolled blood arterial gas, mean arterial blood pressure, heart rate, and rectal temperature (data not shown). No mortality and serious morbidity were observed within the experimental groups. Visual inspection during removal of the brain showed that subarachnoid blood clots had formed and covered the BAs in all animals subject to SAH.

3.2. Tissue Morphometry. The internal elastic lamina in the basilar artery of SAH and SAH+vehicle groups showed substantial corrugation when compared with that obtained from the controls (Figure 1(b)). Corrugation was less prominent

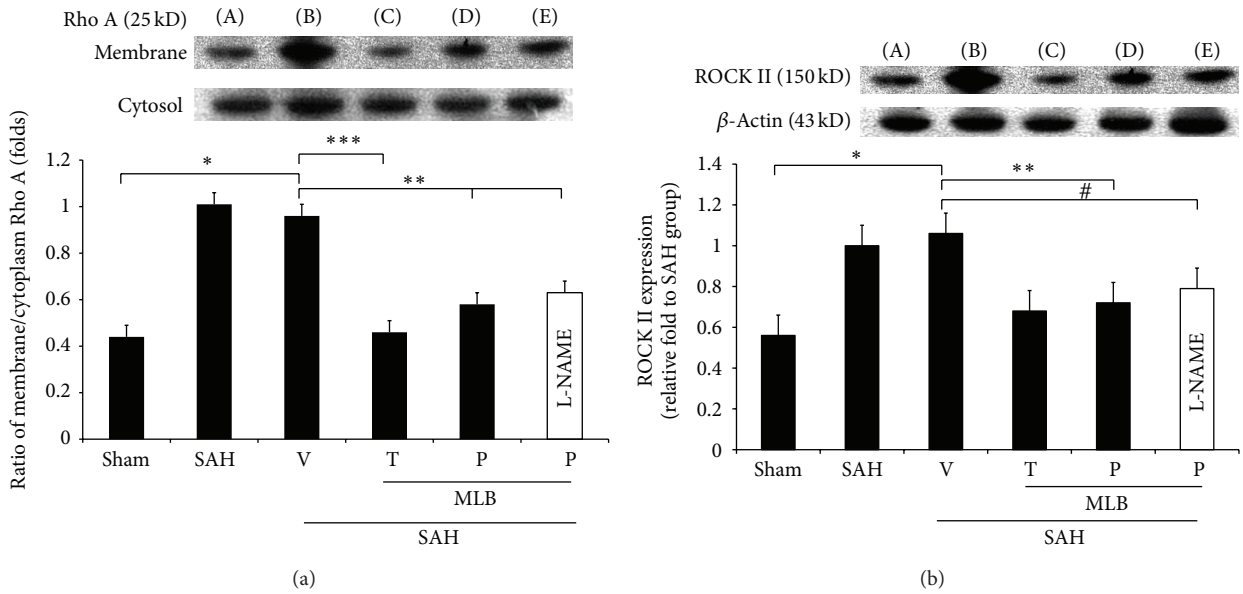

Figure 2: Inhibitory effect of MLB on RhoA translocation (a) and ROCK-II expression (b) in the BAs. Expression of RhoA in the cytoplasm and membrane as well as ROCK-II was also determined by Western blot analysis. (A) the healthy controls, (B) the vehicle-treated SAH rats, (C) SAH rats received 10 mg/kg/day MLB treatment, (D) SAH rats received 10 mg/kg/day MLB preventive treatment, and SAH rats received both 10 mg/kg/day MLB pretreatment and 1 mg/kg L-NAME as (E) (upper panel). The ratio of membrane bound to cytoplasm RhoA in the SAH group was set at 100% in (a), whereas the expression of ROCK-II (normalized using β-actin) was set the same in (b). Data are mean ± SEM (n = 9/group). $^{*,**,***}P < 0.01$, versus the vehicle +SAH, MLB and L-NAME groups, respectively.

in the SAH + 10 mg/kg/day MLB groups. The cross-sectional areas of BAs in the SAH and SAH + vehicle groups were significantly reduced when compared with the control group (0.48 ± 0.27, 0.53 ± 0.18, and 0.93 ± 0.11, resp.). Treatment with MLB significantly attenuated the decrease in both the precondition and reversal groups in previous study.

3.3. Neurological Deficit. Using a modified limb-placing test (MLPT) shown previously, both the scores of ambulation to assess the sensorimotor incorporation of the both forelimb and hindlimb, and placing/stepping reflex as a reflex of response to tactile and proprioceptive stimulation in the SAH groups were significantly higher than the healthy controls. The sum of scores from these two tests is referred to as motor deficit index (MDI). The values of MDI in the SAH and SAH + vehicle groups were 2.88 ± 0.32 and 2.70 ± 0.35, respectively, compared with a score of 0 in the healthy control. Treatment with MLB significantly improved the MDI in the prevention and reversal groups (Table 1). Likewise, paraplegia rate (defined as the percentage of rats with MDI ≥ 3 in each group) was substantially decreased in both the MLB precondition and treatment groups when compared with the SAH animals.

3.4. Expressed eNOS Levels in BAs. When compared with the healthy controls, CSF ET-1 levels in the SAH groups were significantly increased in the pilot study. Treatment with MLB significantly decreased CSF ET-1 in both the MLB prevention and reversal groups to levels that were not aside from the healthy controls. The analysis of radiolabeled L-citrulline showed higher eNOS protein in the control and both MLB prevention and treatment groups. Groups precondition and treatment with MLB tend to increase the expressed eNOS protein in BAs when compared with that in SAH rats ($P < 0.01$; Figure 1(a)). Furthermore, the effect of decreased ET-1 and increased eNOS exerted by MLB was reversed by adding L-NAME (Figure 1(b)).

3.5. RhoA Translocation and ROCK-II Expression in the BAs. Activated rhoA translocated from the cytoplasm to membrane was evaluated. In the BAs, the level of membrane-bound rhoA was significantly increased in rats subject to SAH when compared with the normal control (Figure 2(a)). MLB significantly reduced membrane-bound rhoA, while treatment with vehicle showed no significant difference, when compared with the SAH only group.

The pattern of ROCK-II expression in the BAs resembled that observed with levels of membrane-bound PKCδ or rhoA. ROCK-II expression was significantly increased in the SAH and SAH + vehicle groups when compared with the controls, and treatment with MLB reduced the expression of ROCK-II (Figure 2(b)). The administration of L-NAME significantly increased the bio-expression of rhoA and ROCK-II in the MLB + SAH groups, when compared with the MLB groups (Figure 2).

3.6. Expression of sGC and Associated Downstream cGMP/PKG Pathway. In contrast to the expression of ROCK-II in

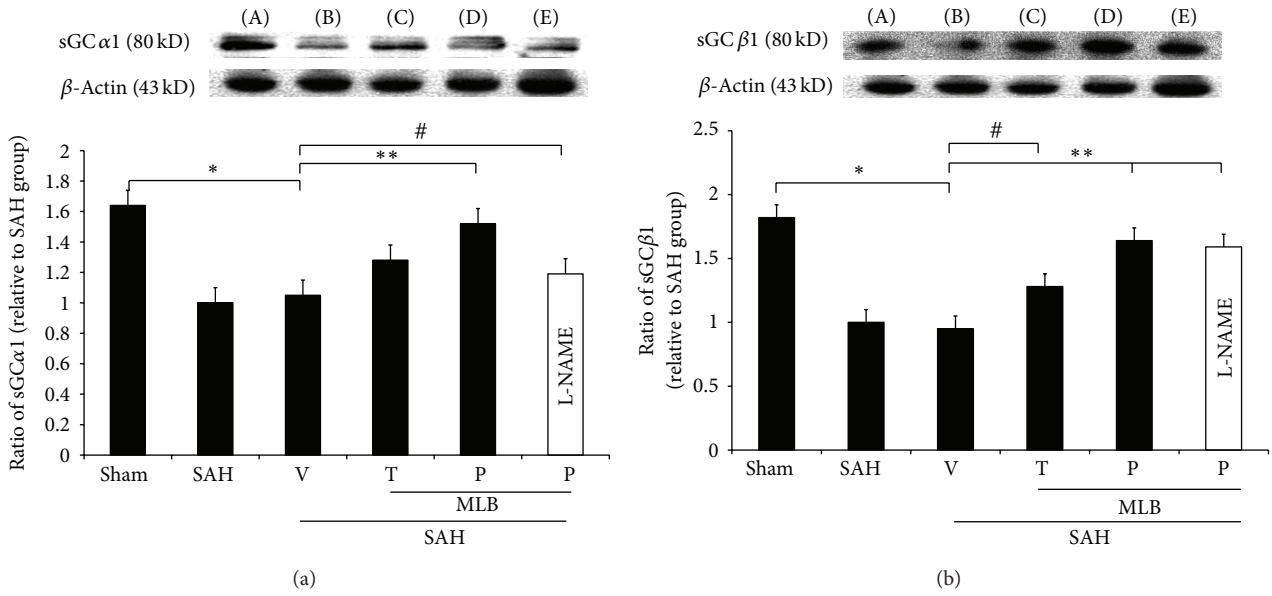

FIGURE 3: Up-regulation of sGCα1 (a) and sGCβ1 (b) by MLB in the BAs. Expression of sGCα1 and sGCβ1 was determined by Western blot analysis and standardized by using β-actin. The expression of sGCα1 and sGCβ1 in the SAH group were set at 100%. All values are mean ± SEM (n = 9/group). $^{*}P < 0.01$, compared with the vehicle + SAH groups. $^{**}0.05 > P > 0.01$, comparison with the vehicle + SAH group, and $^{\#}P > 0.05$, comparison among the vehicle plus SAH, MLB prevention and treatment groups. All groups are equal to those publicized in Figure 2.

the BAs of SAH rats, the level of sGCα1 and sGCβ1 was significantly increased in the MLB pretreatment SAH group compared with the SAH groups, while treatment with MLB failed to reduce sGCα1 and sGCβ1 to a significant level when compared with that in the SAH rats. L-NAME decreased the expressions of these two enzymes to levels similar to those of the SAH groups, which was induced by MLB pretreatment (Figures 3(a) and 3(b)).

Comparative to the reduced sGCα1 and sGCβ1 expressions, the levels of cGMP were significantly decreased in all SAH animals. Administration of MLB in both the prevention and reversal protocols significantly attenuated the reduction of cGMP production in the BAs of the SAH rats (Figure 5). This increased production of cGMP upon MLB treatment also resulted in an increased expression of PKG in the BAs of the SAH rats which, if untreated, showed a significant reduction in PKG expression when compared with the SAH controls (Figure 4). Besides, the administration of L-NAME showed to be effective on the reduction of cGMP level similar to that of SAH groups.

3.7. Translocated PKCδ in Cytoplasm to Membrane. Contrary to that found with rhoA, activated PKCδ was translocated from cytoplasm to membrane. The ratio of membrane to cytoplasm PKCδ expression in the BA of the SAH rats was set as a standard reference. Only half of PKCδ was membrane bound in normal animals; pretreatment with MLB at 1hr after SAH induced the levels of activted PKCδ to the normal control. The treatment of L-NAME reduced MLB's effect on the translocation of activated PKCδ (Figure 4).

4. Discussion

Cerebral vasospasm following spontaneous SAH remains the leading factor contributing to the mortality and morbidity in patients after aneurysm rupture. Even numerous basic and clinical trials conducted, the neurological outcomes for SAH patients are still disappointing [5]. Lines of evidence indicate that both inflammatory and noninflammatory factors are enrolled in the development and maintenance of cerebral vasospasm. Various components of the inflammation have been incriminated in the pathogenesis of cerebral vasospasm, including adhesion molecules [15], cytokines [8], immunoglobulin [8], complement [24, 25], and endothelins [26] at both the cellular and molecular basis. Among them, ET-1, derived from cerebral arteries endothelial cells, has been implicated as a potent vasoconstrictor in mediating SAH-induced vasospasm. Continuous effort pointed to dissect modules besides SAH induced inflammatory response, NO becomes a critical role to mediate vascular dilatation. In the study of Deng et al. [27], small nanomolar concentration of NO, catalyzed by NO synthase, is produced by endothelial cells, which then disperses into vascular smooth muscle (VSM) and acts on the NO-sensitive sGCα1β1 and α2β1. The activation of sGC and another enzyme phosphodiesterases (PDEs), existed in platelets and astrocytes, is involved in the production and maintenance of cGMP. Through activating cGMP-dependent protein kinase (PKG), cGMP is able to mediate the vascular muscle relaxation. The present study showed that MLB significantly improved the motor function index and lowered the rate of paraplegia in the SAH rats. In addition, treatment with MLB significantly increased the

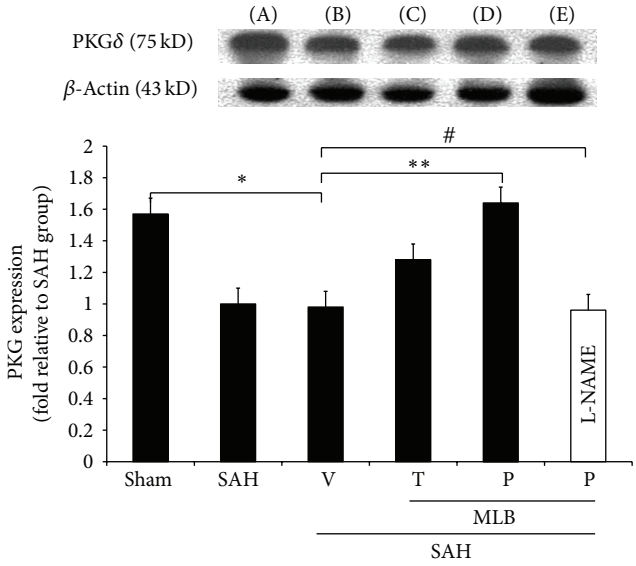

Figure 4: Suppressed PKCδ translocation by MLB in the BAs. The bio-expression of PKCδ from the cytoplasm to membrane was determined by Western blot analysis. The ratio of membrane bound to cytoplasm PKCδ in the SAH group was set at 100%. Data are shown as mean ± SEM (n = 9/group). $^{*,**}P < 0.01$, the vehicle +SAH versus Sham and MLB prevention groups, respectively, and $^{\#}P > 0.05$: compared with SAH rats pre-treatment with MLB and L-NAME. All groups are identical to those shown in the legend of Figure 2.

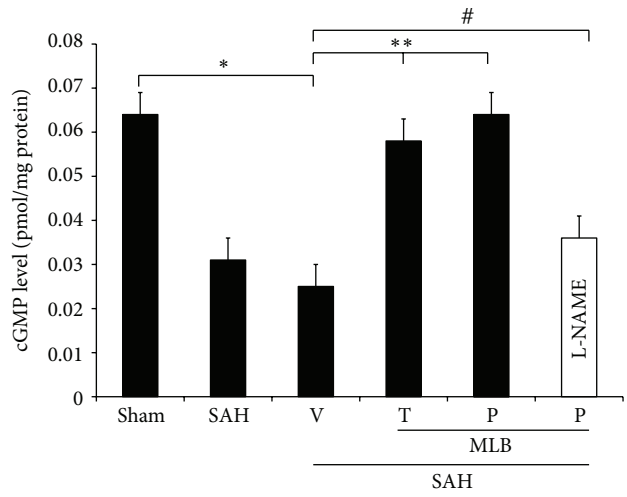

Figure 5: Induced cGMP level by MLB in the BAs. The levels of cGMP (ELISA) was determined. PKG expression in the SAH group was set at 100%. Data are mean ± SEM (n = 9/group). $^{*,**}P < 0.01$, versus the vehicle plus SAH group to the normal controls and MLB groups, respectively. $^{\#}P > 0.05$, comparison among the vehicle plus SAH, L-NAME and MLB prevention group.

levels of eNOs, sGCα1, and cGMP as well as PKG. The addition of NOS inhibitor, L-NAME, decreased the bioexpression of activation of sGCα1, sGCβ1, cGMP, and PKG. These results suggest that the beneficial effects of MLB in cerebral vasospasm after SAH may through additive influence on the NO/sGC/cGMP/PKG pathway.

As described herein, another concept, stated by Brandes et al. [7], indicated through binding to adenosine A2A receptors, activated NOS was able to increase NO and concomitantly disable the PKCδ and rhoA/rho kinase-II pathways in animals subject to SAH. PKCδ and rhoA as well as the expression of rho-kinase were thought to be the main factors contributing to the vascular spastic response along with enhanced components in the sGC/cGMP/PKG pathway. Treatment with MLB induced the activated NOS and normalized the expression of the two vascular regulatory pathways [3]. These results consisted with the reports showing that oxyhemoglobin is a major causative component of blood clot for cerebral vasospasm following SAH [26] and that ET-1 potentiates the oxyhemoglobin-induced cerebrovascular smooth muscle contraction via the rhoA/rho kinase and PKC pathways [28, 29]. In our previous study, MLB can reduce CSF ET-1 level through a NO dependent mechanism [3]. In this study, the level of sGCα1 and sGCβ1 was reduced in the induction of SAH. The administration of L-NAME reverses the enhanced effect of MLB on sGCα1 expression.

In contrast to the activation of the two aforementioned vasoregulatory pathways, SAH resulted in decreased expression of the NO-mediated vasodilatory pathway sGC/cGMP/PKG (Figures 2–4). MLB is an active component extracted from the root of *Salvia miltiorrhiza* (SM). Administration of extract of SM has been shown to attenuate vasoactive mediator and impede the production of ET-1 in patients with congenital heart failure and pulmonary hypertension during cardiac surgery [1, 30]. MLB, as the main component, plays a dual role of antioxidant and potent fibronectin antagonist to holt platelet aggregation, which is able to lessen ischemia associated myocardial damage. In the present study, MLB is proved to be able to improve the endothelial function of vascular wall by suppressing the level of CSF ET-1 and stimulating the sGC/cGMP/PKG pathway via increased production of NO through the inducible NO synthase as seen in the rodent streptozotocin-induced renal injury [2]. In the study of human umbilical vascular endothelial cells (HUVECs), cryptotanshinone, a nonpolar active compound similar to MLB, attenuates the level of ET-1 in the medium as well as the level of TNF-α [30]. This compound is able to inhibit bioactivation of TNF-α-induced NF-κB and is protective to homocysteine-induced endothelium dysfunction [1], and in the other studies, a high dosage cryptotanshinone is able to impede angiotensin converting enzyme (ACE), reduce blood pressure, expand arteries, enhance microcirculation, and albeit atherosclerotic plaque formation [2]. What is more, MLB was reported to protect cerebrum against ischemia reperfusion injury and attenuate the infarct area in a focal cerebral occlusion animal model [1]. Since MLB is composed of a Mg^{2+} cation and a caffeic acid tetramer, the Mg^{2+} salt bridged against MLB is believed to play an adjunct role in blocking excitotoxicity-induced calcium ion influx into smooth muscle cells, which are overstimulated by SAH. Mg^{2+} cation is capable of modulating the L-type calcium channel and augments the

vasodilation effect of NO related pathways in the cardiac smooth muscle [22, 31].

In summary, this study shows that MLB could induce the levels of eNOs, improve the motor function index, activate PKCδ, sGC/cGMP/PKG pathway, and tend to reduce rhoA/rho kinase-II pathway in SAH rats. It is likely that this compound exerts these beneficial effects mainly via its organic caffeic acid tetramer component, which exerts the dual NO related sGC/cGMP/PKG and a G-protein ligand mediated PKCδ pathways. Reduction of rhoA/ROCK II activation through a NO dependent manner also lends evidence to its antivasospastic effect. However, the affinity of MLB to the adenosine A2A receptor needed to be clarified later.

Abbreviations

BA:	Basilar artery
cGMP:	Cyclic -3′,5′-guanosine monophosphate
CSF:	Cerebrospinal fluid
ETs:	Endothelins
GTP:	Guanosine triphosphate,
HRP:	Horseradish peroxidase
IEL:	Internal elastic lamina
i.p.:	Intraperitoneally
LCA:	Leukocyte common antigen
MLB:	Magnesium Lithospermate B
NO:	Nitric oxide
PDEs:	Phosphodiesterases
PKG:	cGMP-dependent protein kinase
PBS:	Phosphate-buffered saline
RhoA:	Ras homolog gene family, member A
ROCK:	RhoA/Rho-kinase
SAH:	Subarachnoid hemorrhage
sGC-α1, -β1:	Soluble guanylyl cyclase -α1, -β1
VSM:	Vascular smooth muscle.

Conflict of Interests

The authors of this paper disclose no financial conflict of interests.

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Research Article

Incidence, National Trend, and Outcome of Nontraumatic Subarachnoid Haemorrhage in Taiwan: Initial Lower Mortality, Poor Long-Term Outcome

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To investigate the longitudinal trend of nontraumatic subarachnoid haemorrhage (SAH), we analyzed the annual population-based incidence and mortality rate of nontraumatic subarachnoid hemorrhage in Taiwan. Logistic regression was used to identify independent predictors of mortality. The average incidence rate (IR) of nontraumatic SAH was 6.25 ± 0.88 per 100,000 per year. The prevalence of female patients was higher than in the male population (54.5% versus 45.5%). The average age of these patients was 55.78 ± 17.09 and females were older than males (58.50 ± 15.9 versus 52.45 ± 18.50, $P < 0.001$). Of these patients, 97.6% (611/626) were treated with surgical intervention with clipping procedure and 2.9% (18/626) with coiling. Total mortality of these patients was 13.4% (84/626). In adjusted analysis, age (odds ratio [OR], 0.97; 95% confidence interval [CI], 0.98-0.98; $P < 0.001$) and Charlson comorbidity index (OR, 0.709; 95% CI, 0.57–0.88; $P = 0.002$) remained independent predictors of the mortality. Patients with nontraumatic SAH had a much higher prevalence in older age groups and in females than in the general population. Patients with old age and more comorbidity have higher mortality. Aggressive management of patients might reduce the initial mortality; however, patient outcome still remains poor.

1. Introduction

Nontraumatic subarachnoid hemorrhage (SAH) is a critical and serious disease. After rupture, patients usually suffer severe headache and consequent loss of consciousness. According to previous studies, countries with high incidence of around 20 per 100,000 person years (PY), such as Finland and Japan, and countries with low incidence of approximately 5–10 per 100,000 PY in The Netherlands [1, 2]. Significant variation in incidence rates between countries are reported for nontraumatic SAH [3]. In the studies from Europe, there might be existing regional differences [4–7]. Seasonal variation, diurnal, and daily factors were also found to be associated with the incidence of nontraumatic subarachnoid hemorrhage [8, 9].

Most patients found to have that this disease occurred suddenly without portend. In previous studies, nontraumatic SAH was found to affect more females and the older population [2, 6, 7, 10]. Global mortality ranges from 32 to 67% [11] with around 20% of SAH patients dying before arriving at the hospital [12]. Most of the nontraumatic SAH needed emergency intervention, such as aneurysm clipping or angioembolization with coil [13]. Endovascular coiling as compared to neurosurgical clipping had better general clinical outcomes [14, 15]. Hypertension, smoking, high alcohol

intake, and extreme physical exertion were considered as risk factors of SAH [16, 17].

Many developed countries are facing the challenge of an aging population. With an increasingly aging population, the occurrence of nontraumatic SAH will predictably rapidly increase and will represent a major and growing health care problem. Thus, with a very large variation in incidence of nontraumatic subarachnoid hemorrhage in this region and many countries lacking comprehensive data and available reports, we conducted this study to investigate the trend of epidemiology and outcome of nontraumatic SAH from 2000 to 2009 in Taiwan and compared our result with other countries.

2. Methods

2.1. Database. Taiwan implemented an NHI program in March 1995, offering a comprehensive, unified, and universal health insurance program to all citizens. We conducted a nationwide survey of nontraumatic SAH in Taiwan from 2000 to 2009 based on the National Health Insurance Research Database. This cohort dataset comprised 1,000,000 randomly sampled beneficiaries still enrolled in the NHI program during 2000 and collected all records on these individuals from 1995 to 2009 containing basic demographic information on insured residents (sex, age, region, and so on), along with medical records (including inpatient and ambulatory visits). Healthcare facilities contracted under the NHI provide the insured of the NHI with inpatient care, ambulatory care, dental care, and prescription drugs. The claims data of the NHI are routinely monitored by the Bureau of the NHI for their accuracy and completeness. According to the Taiwan National Health Research Institute, there were no statistically significant differences in age, gender, or health care costs between the sample group and all beneficiaries under the NHI program. The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University.

2.2. Study Sample. The International Classification of Disease, Ninth Revision (ICD-9) to define nontraumatic SAH was used to claim the data. From the NHI inpatient database, we identified people newly diagnosed with ICD-9-CM codes from 2000 to 2009 with codes: 430.0 (ruptured aneurysm), 430 (subarachnoid hemorrhage), and 437.3 (unruptured aneurysm) of brain aneurysm and ICD-9 codes. The treatment codes with 39.51 (clipping of aneurysm), 39.79, 39.72, 39.25 (coiling), 38.31 (suture of artery), 39.52 (other repair of aneurysm), 38.81, and 38.82 (other surgical occlusion of intracranial vessels) were also used for inclusion criteria. The exclusion criteria included 748.8 (arteriovenous malformation). Because of this financial incentive, almost everyone with aneurysm in Taiwan is included in the health system; thus, the identification of brain aneurysm from the database should be nearly 100%. In addition, all the diagnoses and management of brain aneurysm were by neurosurgeons in Taiwan; thus, the brain aneurysm cases identified from the dataset should be accurate. Mortality rate was defined within 30 days of hospital admission. Because Taiwan's NHI is the only payer health program, there is no other reason for being withdrawn from the NHI coverage within 30 days of hospital admission expect for patient mortality. Overall mortality is estimated if patients do not expire over more than 3 months but expire during the study period. The coding algorithm reported by Deyo et al. was used to identify 17 prognostic comorbidity conditions and calculate the Charlson comorbidity index (CCI) according to specific weights assigned to each condition [18, 19].

2.3. Statistical Analysis. Mean, median, and interquartile range (IQR) were generated for continuously coded variables. Frequencies and proportions were generated for categorical variables. The categorical data between the two groups were compared with chi-square test and Fisher exact test as appropriate. Comparisons between continuous variables were done by Student's t-test. One-way ANOVA rated the difference of age between years and disease occurrences between seasons. Subsequently, binary unconditional logistic regression models were fitted to test the effect of age, gender, and CCI on the mortality. Statistical significance was inferred at a 2-sided P value of <0.05. All statistical analyses were carried out using the Statistical Package for Social Science, version 19.0 (SPSS, Chicago, IL).

3. Results

Overall, 627 patients with nontraumatic SAH were identified in the database. One patient with missed data of gender was excluded. The average incidence rate (IR) of nontraumatic SAH was 62.5 ± 8.82 per 1,000,000 per year. There was no significant association between gender and years (chi-square test; $P = 0.280$) (Figure 1). The prevalence of female patients was higher than that of males (54.5% versus 45.5%).

Overall, total mortality of these patients was 13.4% (84/626). The mortality of each year showed differences ($P = 0.06$). There was a trend of increasing mortality from 2000 to 2009 (Figure 2). However, there was no difference of mortality between gender (females versus males: 13.7% [47/344] versus 12.8% [37/288]; $P = 0.885$) and performing of tracheostomy (Yes versus No 7.8% [4/51] versus 6.4% [37/574]; $P = 0.699$).

The average age of these patients was 55.78 ± 17.09 (Figure 3). Females found to have the disease were older than males similarly affected ($P < 0.001$). Nonsurviving patients were older on average than those surviving (61.95 ± 17.23 versus 55.30 ± 17.02; $P = 0.016$). The distribution of patient's age had no difference from 2002 to 2009 (one-way ANOVA; $P = 0.209$). In adjusted analysis, age (odds ratio [OR], 0.97; 95% confidence interval [CI], 0.98-0.98; $P < 0.001$) and Charlson comorbidity index (OR, 0.709; 95% CI, 0.57–0.88; $P = 0.002$) remained as independent predictors of mortality.

There were no differences of disease occurrence between seasons (one-way ANOVA; $P = 0.661$). Of these patients, 97.6% (611/626) were treated with surgical intervention with clipping procedure and 2.9% (18/626) with coiling. For treatment location, 70.4% (441/626) patients were treated

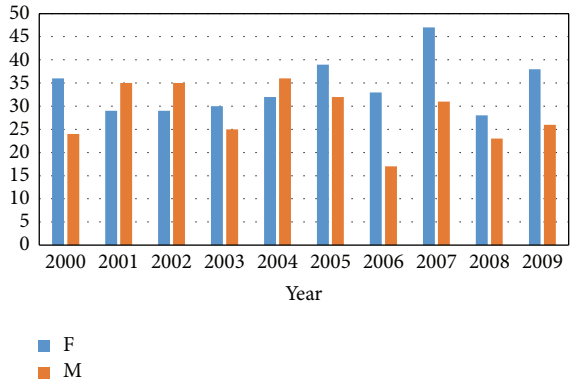

Figure 1: The percentage of a total 625 patients from 2000 to 2009 with no trend of increasing or decreasing patient number.

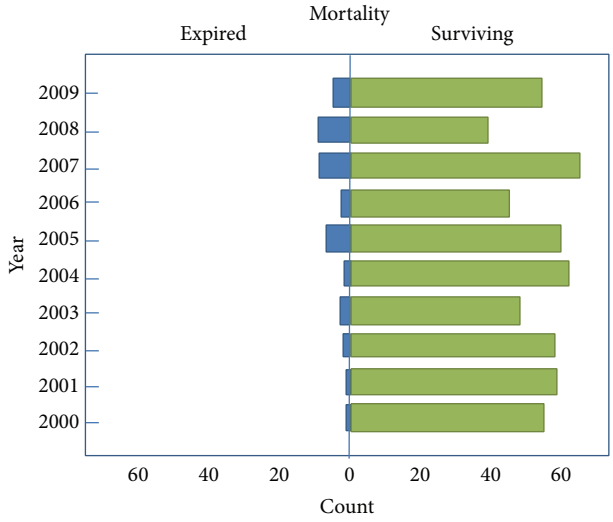

Figure 2: The trend of mortality from 2001 to 2009.

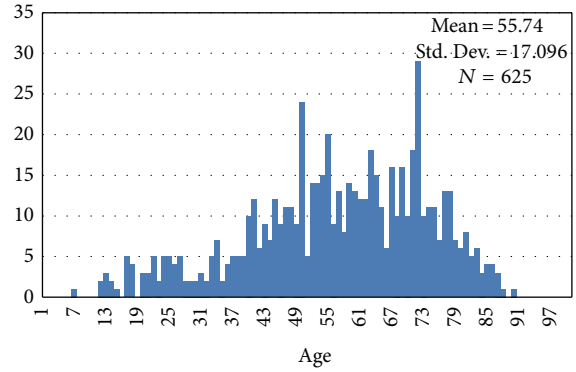

Figure 3: The distribution of age in patients having nontraumatic subarachnoid haemorrhage.

at medical centers; 27.3% (171/626) patients were treated at regional hospitals; and 2.2% (14/626) patients were treated at district hospitals. During the study period, the overall survival rate of these patients was 71.9% (455/626).

4. Discussion

In this study, we found that patients with nontraumatic SAH had a much higher prevalence in older age groups and in females than in the general population. Toward the end of the study period, similar overall outcomes were observed in the trend of incidence and mortality. When compared to other countries, incidence in Taiwan was similar to other countries but the initial mortality rate was lower.

The 30-day case-fatality rate of the hospitalized nontraumatic SAH was high with range from 30% to 34.7% [1, 7, 20]. In the previous studies, the median case fatality rate in Europe was above 40%; in Asia it was about 35.8% except for Japan at 26.7%; in South America and the Caribbean it was 32.5%; in Australia and New Zealand it was 41.7%; and in the USA it was 32.2% with publications between 1965 and July 2007, and the period of survey was later than 1960 [21]. In our study, we found that the mortality rate was lower than other countries (13.4%). However, operative rate was higher in our study group. The medical policy in Taiwan allows most patients to receive intensive care with surgical intervention without worrying about the medical expense. The higher surgical rate is because the financial policies do not pay for coiling; this impacts the treatment decision for patients during the study period. With aggressive medical treatments, mortality may be reduced and become lower than in other countries. In addition, 97.4% patients of these patients were treated at medical centers and regional hospitals. Surgical intervention of brain aneurysm requires a skillful neurosurgeon and these facilities have staff capable of managing these patients; therefore, the lower mortality may result from easy accessibility of medical resources.

There are few studies of the incidence of nontraumatic aneurysm in Asia. Although the aggressive treatment of the aneurysm may result in lower mortality within 30 days; however, the overall mortality reaches 30% eventually. The health system in Taiwan provides relatively better financial support and patients can receive aggressive treatment including 97.6% of patients being treated with surgical intervention without consideration of medical expense, which may improve the short-term survival rate. In addition, most of the EMTs can deliver a critical patient to a hospital within 30 minutes, and this can decrease secondary brain injury after a stroke episode. Therefore, the health providing systems may rather influence the short-term outcome of patient mortality. Similar results of lower mortality can be found in the studies from Japan, where the medical system is similar to Taiwan. Our study showed a trend of increase in incidence and mortality rates of aneurysmal SAH in Taiwan. After 2000, the Hospice Palliative Act was implemented. Some of these patients might give up after finding poor outcome [22], which might increase the mortality within 30 days after the implementation of the law.

Age was found to be a predictor of poor outcome [7]. In this study, we found that age and Charlson comorbidity index remained independent predictors of mortality. Older people have more comorbidity than do young people. There was no

statistical difference of mortality between seasons, which may result from the tropical location of Taiwan where temperature change is not obvious related to seasonal change. Females are prone to have nontraumatic SAH; however, their average age was older than that for males. The protective effect may result from hormonal influence before menopause [23, 24].

The strengths of this study include its nationwide population and the large number of patients included in the analysis. Based on observational data, the results of our study should be interpreted in the light of potential limitations, such as selection bias and information bias. First, in common with other studies using administrative databases, no information on nontraumatic SAH severity was available for risk adjustment. Patients who died before reaching hospital were not included in the NHI estimate, and it is highly likely that they were also missed in the NHI database due to lack of proper diagnosis and specialist information. This means that the true incidence is potentially higher than in our study. Previous studies show that the percentage of persons dying before reaching hospitals was between 11 and 13% [7, 12]. Second, the selection of variables was limited because the data source was a secondary one. Because of lack of information on processes of care, we could not identify the unmeasured variables that might explain the differences in nontraumatic SAH mortality. Third, nontraumatic SAH diagnoses are based on hospitals' claims, so the accuracy of nontraumatic SAH coding could be questioned. However, it must be noted that the NHI regularly and randomly samples a percentage of cases from hospitals to verify the validity of diagnosis and quality of care through chart reviews using touring professional teams, and all the diagnosis of the codes were by the neurosurgeons. We think this error is limited in the NHI database as we applied a very sensitive search on codes. Further prospective study focused on the predictor factors of patients deemed to be prone to mortality may decrease futile treatment. If further advance of intervention such as angioembolization can provide better outcome, aggressive treatment might provide more opportunity for patient survival.

5. Conclusion

Patients with old age and more comorbidity have higher mortality. In Taiwan, the initial mortality rate of nontraumatic aneurysm is lower as the health provision system might improve incidence of short-term mortality; however, the overall mortality was found to be similar to other countries. Aggressive management of patients might reduce the initial mortality; however, patient outcome still remains poor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hsing-Lin Lin and Kwan-Ming Soo contributed equally to this paper and are considered as first authors. Hsing-Lin Lin and Kwan-Ming Soo were responsible for conception and design. Chao-Wen Chen was responsible for data acquisition. Yen-Ko Lin was responsible for the literature search. Chao-Wen Chen and Hsing-Lin Lin were responsible for statistical analysis. Hsing-Lin Lin and Shiuh-Lin Huang were responsible for drafting the paper. Wei-Che Lee and Shiuh-Lin Huang were responsible for critical revision of the paper. All authors gave final approval.

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Clinical Study

Cerebral Vasospasm in Patients over 80 Years Treated by Coil Embolization for Ruptured Cerebral Aneurysms

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Object. The effect on clinical outcomes of symptomatic vasospasm after aneurysmal subarachnoid hemorrhage (SAH) in patients over 80 years who underwent coil embolization was evaluated. *Methods*. Forty-four cases were reviewed and divided into two groups according to patient age: Group A, 79 years or younger, and Group B, 80 or older. Patient characteristics, prevalence of symptomatic vasospasm, modified Rankin Scale (mRS) scores at discharge and frequency of symptomatic vasospasm in patients with mRS scores of 3–6 were analyzed. *Results*. Thirty-two (73%) of the 44 cases were categorized as Group A and 12 (27%) as Group B. Group B had a significantly higher prevalence of symptomatic vasospasm compared to Group A ($P = 0.0040$). mRS scores at discharge were significantly higher in Group B than in Group A ($P = 0.0494$). Among cases with mRS scores of 3–6, there was a significantly higher frequency of symptomatic vasospasm in Group B than in Group A ($P = 0.0223$). *Conclusions*. In our cohort of aneurysmal SAH patients treated by coil embolization, patients over 80 years of age were more likely to suffer symptomatic vasospasm, which significantly correlated with worse clinical outcomes, than those 79 years and under.

1. Introduction

The numbers of patients over 80 years of age with subarachnoid hemorrhage (SAH) due to rupture of cerebral aneurysms are increasing. According to the Japanese Stroke Data Bank, a database on acute stroke patients with the aim of furthering the standardization of stroke management in Japan, about 10% of SAH patients in 2009 were over 80 years of age. The International Subarachnoid Aneurysm Trial (ISAT) revealed the superiority of coiling to clipping for ruptured cerebral aneurysms in terms of clinical outcomes [1]. Coil embolization is also less invasive, and its advantages for elderly patients have already been reported [2–5]. Together, these findings are increasing the popularity of coil embolization and also the likelihood that a patient treated for ruptured cerebral aneurysm will undergo coil embolization.

Cerebral vasospasm is the most common cause of focal ischemia after SAH and has been reported to be one of the important causes of death and disability among SAH patients [6]. A few papers have investigated the effect of age on the occurrence of cerebral vasospasm in surgically treated cases [7, 8]. In the present study, we evaluated the effect of vasospasm on outcomes in patients who had undergone coil embolization for ruptured cerebral aneurysm at our institute, comparing patients over 80 years of age with those 79 years and under.

2. Patients and Methods

2.1. Patients. We reviewed the clinical information of all patients admitted to Okayama University Hospital with acute aneurysmal SAH from January 2003 to May 2012. We included 88 patients who met the following inclusion criteria: (1) SAH as demonstrated on computed tomography (CT); (2) evidence for ruptured intracranial aneurysm as demonstrated by cerebral angiography or 3-dimensional CT angiography; and (3) aneurysm treated with surgical clipping

or endovascular coiling. Decisions regarding aneurysm treatment modality were made on the basis of factors including aneurysm location, size and shape, patient age, neurological grading (Hunt and Kosnik grade) [9], and Fisher group [10]. Finally, the medical records of 44 aneurysmal SAH patients treated by coil embolization were evaluated retrospectively. Two patients experienced two episodes of SAH during this period. Only the first SAH of these two patients was included and a total of 44 aneurysmal SAH cases were analyzed in this study. These cases were divided into two groups, according to patient age: Group A, 79 years or younger, and Group B, 80 years or older.

2.2. Endovascular Procedures. All endovascular procedures but one were performed under general anesthesia. The simple technique was used in 23 cases (52%); the other 21 (48%) were treated using adjunctive techniques as follows: balloon remodeling technique (BRT): 15 cases (34%), double catheter technique (DCT): 3 cases (7%), BRT combined with DCT: 3 cases (7%). Two senior neurosurgeons (K. Sugiu and K. Tokunaga) performed all endovascular procedures.

2.3. Management of Cerebral Vasospasm. All cases were maintained in a normotensive and normovolemic state and treated with thromboxane A_2 synthesis inhibitor and fasdil chloride intravenously starting immediately after coil embolization. After the diagnosis of symptomatic vasospasm had been reached, mild hypertensive hypervolemic therapy was initiated. Lumbar drainage was indicated for the patients assigned to Fisher group 3 and inserted in 36 cases (82%) to remove subarachnoid clots. Symptomatic vasospasm was defined, according to Shirao et al. [11], as (1) the presence of neurological worsening including focal deficit, decline in level of consciousness, and motor paresis; (2) no other identifiable cause of neurological worsening; (3) confirmation of vasospasm by medical examinations including evidence of vasospasm by radiographic assessment. In our institute, symptomatic vasospasm was confirmed by angiography and subsequently treated with endovascular intra-arterial fasdil chloride administration and/or angioplasty. To identify any instances of cerebral infarction, patients with symptomatic vasospasm were repeatedly checked by CT scanning. There were no differences of the treatment for vasospasm between the groups.

2.4. Neurological Evaluation. A neurosurgeon in our institute assessed the neurological status of all patients using the modified Rankin Scale (mRS) [12] at the time of hospital discharge.

2.5. Statistical Analysis. Quantitative variables are presented as percentages or as medians and interquartile ranges (IQRs). Statistical analysis was performed using Fisher's exact probability test, the chi-square test, and the Mann-Whitney U test, as appropriate. All statistical analyses were performed using StatView (SAS Institute, Cary, NC, USA). Differences were considered to be significant when P values were less than 0.05.

3. Results

Thirty-two (73%) of the 44 cases were categorized as Group A and 12 (27%) as Group B. Table 1 shows the patient characteristics of the cases in this study. There was no statistically significant difference related to patient sex or treatment day. Group B had a tendency toward more anterior circulation aneurysms compared to Group A, but this was not significant ($P = 0.0679$). Fisher groups and Hunt and Kosnik grades were evenly distributed in Groups A and B.

3.1. Prevalence of Symptomatic Vasospasm and Cerebral Infarction. The frequency of insertion of lumbar drainage was significantly higher in Group A than in Group B ($P = 0.0249$). Four of the 32 cases (13%) in Group A and 7 of the 12 cases (58%) in Group B exhibited symptomatic vasospasm. The difference in the prevalence of symptomatic vasospasm between Groups A and B was statistically significant ($P = 0.0040$). Group B had a significantly higher prevalence of cerebral infarction due to vasospasm compared to Group A (33% versus 3%, $P = 0.0153$).

3.2. Outcomes at Discharge. mRS scores at discharge were significantly higher in Group B than in Group A (median 4 [IQR 3.75–4.25] versus 3 [IQR 1.75–4], $P = 0.0494$, Table 1). There was no significant difference between Groups A and B in length of hospital stay (Table 1).

3.3. Effect of Symptomatic Vasospasm on Outcomes. Table 2 shows the prevalence of symptomatic vasospasm in patients with mRS scores of 3–6 at discharge in Groups A and B. Four of 23 cases (17%) with mRS scores of 3–6 in Group A and 7 of 12 cases (58%) with mRS scores of 3–6 in Group B exhibited symptomatic vasospasm. There was a significant difference between Groups A and B in the frequency of symptomatic vasospasm in cases with mRS scores of 3–6 ($P = 0.0223$).

4. Discussion

4.1. Aging and SAH. As Japan has the world's highest life expectancy, aging in stroke patients has become an important social issue from the perspective of health care and medical economy in Japan. The incidence of SAH increases with age, and this tendency is of peculiar note given the recent aging of the population. Inagawa [13] reported that the percentage of very elderly SAH patients over 80 years in Izumo city, Japan, increased from 5% in 1980–1989 to 18% in 1990–1998. It has also been reported that the size of ruptured aneurysms tends to increase with age [14]. Considering the larger size of aneurysms and the likely presence of comorbid disease in patients over 80 years, determining optimal management plans for these patients is difficult, and the prognosis of elderly SAH patients has been reported to be poor [8]. Recently, coil embolization is becoming a more common treatment for aneurysmal SAH patients, especially elderly patients, as this treatment has been proven to contribute to better clinical outcomes in comparison with clipping in a large randomized clinical trial [1]. It would be helpful to elucidate the outcomes of aneurysmal SAH patients over 80

Table 1: Patient characteristics.

	No. of cases (%)		*P* value
	Group A (32 cases)	Group B (12 cases)	
Age, yr (median, IQR)	64 (58.75–69.75)	83 (82–84.25)	
Sex (male : female)	12 : 20	6 : 6	0.4526
Treatment day (median, IQR)	0 (0-1)	0 (0-1)	0.9877
Location of aneurysms (anterior circulation)	19 (59)	11 (92)	0.0679
Fisher groups			0.1158
2	2 (6)	3 (25)	
3	30 (94)	9 (75)	
Hunt and Kosnik grades			0.4752
I	3 (9)	3 (25)	
II	8 (25)	3 (25)	
III	11 (35)	2 (17)	
IV	10 (31)	4 (33)	
Insertion of lumbar drainage	29 (91)	7 (58)	0.0249
Symptomatic vasospasm	4 (13)	7 (58)	0.0040
Cerebral infarction	1 (3)	4 (33)	0.0153
Length of stay, days (median, IQR)	30 (17–53)	32.5 (14.75–50.25)	0.7515
mRS score at discharge			0.0494
0	2 (6)	0 (0)	
1	6 (19)	0 (0)	
2	1 (3)	0 (0)	
3	9 (28)	3 (25)	
4	9 (28)	6 (50)	
5	5 (16)	3 (25)	
6	0 (0)	0 (0)	

Age, treatment day, and length of stay values represent medians (interquartile range); other values represent raw numbers with percentages in parentheses. mRS indicates modified Rankin Scale.

Table 2: Prevalence of symptomatic vasospasm in patients with mRS score of 3–6 at discharge.

	Group A (23 cases)	Group B (12 cases)	*P* value
Symptomatic vasospasm			
Yes	4 (17)	7 (58)	0.0223
No	19 (83)	5 (42)	

Values represent raw numbers with percentages in parentheses.

years of age who have been treated by coil embolization, but few studies have analyzed outcomes in this population exclusively. This is the first report examining the effect of vasospasm on clinical outcomes in patients over 80 years of age treated by coil embolization.

4.2. Significance of Vasospasm in Elderly SAH Patients. This study revealed that clinical outcomes at discharge were significantly worse in patients over 80 years than in patients 79 years and under. The overall prevalence of symptomatic vasospasm was significantly higher in patients over 80 years than in patients 79 years and under (58% versus 13%). In addition, the frequency of symptomatic vasospasm in patients with poor outcome (mRS scores 3–6) was significantly higher in patients over 80 years than in patients 79 years and under (58% versus 17%).

Several mechanisms could be responsible for the higher prevalence of symptomatic vasospasm among elderly patients. First, aging itself could be related to the higher prevalence of symptomatic vasospasm in elderly patients. Inagawa [15] demonstrated that angiographical vasospasm in major cerebral vessels tended to be lower in the elderly because of good clearance of the accumulated blood clots with cerebrospinal fluid or atherosclerotic changes of arteries. Other papers have reported that elderly patients developed slightly but significantly fewer angiographical vasospasms than younger patients did, although they tolerated ischemia less well and were prone to develop infarction [7, 8]. In fact, our data reveal a significantly higher prevalence of cerebral infarction in patients over 80 years of age than in patients of 79 years and under. Together with the data from previous reports [7, 8, 15], our data indicates that there is a discrepancy between angiographical vasospasm and cerebral ischemia in elderly patients; our data also raise the possibility that

the discrepancy could be attributable to the vulnerability of the aged brain. This vulnerability of the aged brain to vasospasm is probably explained by the reduction of cerebral blood flow or the impairment of vascular reserve in elderly SAH patients [16, 17]. Especially in patients over 80 years of age, aging has a profound effect on symptomatic vasospasm and subsequent cerebral infarction. Second, the effect of lumbar drainage on vasospasm should be considered. Klimo Jr. et al. [18] reported that shunting of cerebrospinal fluid through a lumbar drainage after SAH markedly reduced the risk of vasospasm and improved outcomes. The lower frequency of insertion of lumbar drainage in patients over 80 years in this investigation might be related to the higher prevalence of symptomatic vasospasm. It is generally supposed to be more difficult to insert lumbar drainage in patients over 80 years of age in the clinical setting because they sometimes have significant lumbar spine degeneration.

The overall correlation of poor outcome with advancing age has been partially explained by some factors, such as mRS scores at onset [19], brain atrophy [20], poor neurological grade at admission [21], increased amount of blood on CT, and preexisting medical diseases [8]. The greater incidence of poor outcome in elderly patients is multifactorial, and symptomatic vasospasm is probably just one of the factors involved in poor outcome. Of the various factors that can be involved, vasospasm is significant as the only pathophysiology that can be overcome through successful perioperative management. Cerebral vasospasm even in SAH patients over 80 years of age should be treated more aggressively and earlier to improve clinical outcomes.

4.3. Study Limitations. There are certain limitations to this study that should be noted. First, this study had a retrospective nature and a limited sample size. A larger population of SAH patients is needed to build the accurate evidence on the effect on clinical outcomes of symptomatic vasospasm in patients over 80 years who underwent coil embolization. Second, the outcomes of patients over 80 years of age in our investigation were worse than those in some previous reports [22, 23]. This is probably because the length of hospital stay was relatively short (median 32.5 days) and outcomes were therefore evaluated relatively early. A longer follow-up in a future study is warranted because some patients could improve their mRS scores with the aid of rehabilitation. Third, the patients in this study were not randomized; rather, there was a selection bias involving in determining treatment modality. Patients over 80 years of age tended to have more anterior circulation aneurysms compared to patients 79 years and under in this investigation. Yet there is no definitive evidence of a significant relationship between aneurysm location and symptomatic vasospasm [24, 25]. The difference between the groups in aneurysm location probably had little influence on the occurrence of symptomatic vasospasm.

5. Conclusions

In our patient cohort, aneurysmal SAH patients treated by coil embolization who were over 80 years of age were more likely to suffer symptomatic vasospasm compared to those of 79 years and under. In patients over 80 years, symptomatic vasospasm was significantly correlated with worse clinical outcomes. The development of an improved treatment for vasospasm could help improve clinical outcomes in SAH patients over 80 years of age.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Therapeutic Implications of Estrogen for Cerebral Vasospasm and Delayed Cerebral Ischemia Induced by Aneurysmal Subarachnoid Hemorrhage

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Cerebral vasospasm (CV) remains the leading cause of delayed morbidity and mortality following aneurysmal subarachnoid hemorrhage (SAH). However, increasing evidence supports etiologies of delayed cerebral ischemia (DCI) other than CV. Estrogen, specifically 17β-estradiol (E2), has potential therapeutic implications for ameliorating the delayed neurological deterioration which follows aneurysmal SAH. We review the causes of CV and DCI and examine the evidence for E2-mediated vasodilation and neuroprotection. E2 potentiates vasodilation by activating endothelial nitric oxide synthase (eNOS), preventing increased inducible NOS (iNOS) activity caused by SAH, and decreasing endothelin-1 production. E2 provides neuroprotection by increasing thioredoxin expression, decreasing c-Jun N-terminal kinase activity, increasing neuroglobin levels, preventing SAH-induced suppression of the Akt signaling pathway, and upregulating the expression of adenosine A2a receptor. The net effect of E2 modulation of these various effectors is the promotion of neuronal survival, inhibition of apoptosis, and decreased oxidative damage and inflammation. E2 is a potentially potent therapeutic tool for improving outcomes related to post-SAH CV and DCI. However, clinical evidence supporting its benefits remains lacking. Given the promising preclinical data available, further studies utilizing E2 for the treatment of patients with ruptured intracranial aneurysms appear warranted.

1. Introduction

Spontaneous subarachnoid hemorrhage (SAH) secondary to rupture of intracranial aneurysms represents a relatively small fraction of strokes (5%). However, the morbidity and mortality associated with aneurysm rupture remain very high despite advances in the diagnosis and treatment of aneurysmal SAH [1]. Cerebral vasospasm (CV) is the leading cause of delayed morbidity and mortality following aneurysmal subarachnoid hemorrhage (SAH). While radiographic CV is present in up to 70% of SAH patients, clinically symptomatic CV occurs in only 20–30% [2]. However, it is now evident that CV alone is inadequate to completely explain the delayed neurological dysfunction which occurs in the one to two weeks following the ictus of aneurysm rupture [3, 4].

After the age of 40, aneurysmal SAH is more common in females [5, 6]. The typical age of menopause, when serum estrogen levels decrease dramatically, is approximately 50 years [7]. It remains controversial whether the temporal relationship between decreased estrogen levels and increased

incidence of aneurysm rupture in women is causative [8]. Despite extensive research on the effect of estrogen on aneurysm formation, progression, and rupture, the efficacy of estrogen for the treatment of SAH-induced CV has not been well investigated. With this deficiency in mind, we review the pathogenesis of aneurysmal SAH-induced CV, delayed cerebral ischemia (DCI), and the potential role of estrogen, specifically 17β-estradiol (E2), in combating these two interrelated but distinct cerebrovascular disease processes.

2. Pathogenesis of Aneurysmal Subarachnoid Hemorrhage-Induced Cerebral Vasospasm

2.1. Smooth Muscle Cell Contractile Mechanisms. In order to properly understand the molecular mechanisms of CV, we first briefly describe the manner by which smooth muscle cells (SMC) in the media of cerebral vasculature regulate contraction and relaxation [9]. The process of SMC contraction begins with opening of voltage-gated or ligand-gated calcium (Ca) channels allowing the entry of Ca from the extracellular space and from within the sarcoplasmic reticulum into the cytoplasm. The binding of cytoplasmic Ca to calmodulin (CaM), forming the Ca-CaM complex, can then activate the enzyme myosin light chain kinase (MLCK) to phosphorylate myosin. Phosphorylation of myosin allows it to bind to actin. At a cellular level, the coupling of myosin to actin results in SMC contraction which translates to vasoconstriction at the physiologic level.

2.2. Mechanisms of Cerebral Vasospasm Secondary to Endothelial Injury. Endothelial dysfunction is one of the primary contributing factors to CV following aneurysmal SAH. Endothelins (ET) are the most potent endogenous activators of vasoconstriction. ETs are produced by the endothelium and play a key role in maintaining vascular homeostasis. In the setting of SAH, the cerebrospinal fluid (CSF) levels of ET-1, the most common ET isoform, have been shown to be increased [10]. ET-1 binds to the endothelin receptor of which the two best characterized and most studied isoforms are ET_A and ET_B [11]. Binding of ET-1 to ET_A and ET_B results in vasoconstriction via pathways mediated by protein kinase C (PKC) [12]. PKC, which is activated by diacylglycerol (DAG) generated by phospholipase C and Ca generated from the opening of inositol triphosphate (IP3) gated Ca channels in the sarcoplasmic reticulum, promotes further Ca influx by opening cell surface Ca channels. In addition to Ca-dependent vasoconstriction, PKC also mediates Ca-independent vasoconstriction and vascular remodeling via mitogen-activated protein kinase (MAPK) [13].

Nitric oxide (NO), also known as endothelium-derived relaxing factor, is produced from arginine by the enzyme nitric oxide synthase (NOS) which exists in constitutively expressed isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS), and the inducible isoform iNOS [14]. NO induces SMC relaxation increasing intracellular levels of the second messenger cyclic guanine monophosphate (cGMP) via activation of guanylate cyclase. The mechanisms by which elevated cGMP levels promote vasodilation include prevention of SMC depolarization by inhibition of Ca influx, facilitation of SMC hyperpolarization by activation of potassium (K) channels, and inhibition of SMC contraction by dephosphorylation of myosin by myosin light chain phosphatase (MLCP) which is activated by a cGMP-dependent kinase [15]. Decreased availability of NO secondary to hemoglobin-mediated destruction of nNOS and endogenous asymmetric dimethylarginine (ADMA) mediated inhibition of eNOS also contributes to the development of CV [16].

2.3. Mechanisms of Cerebral Vasospasm Secondary to Inflammation and Smooth Muscle Cell Injury. Acute subarachnoid hemorrhage generates the product oxyhemoglobin (oxy-Hb) which results in vasoconstriction via generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide [17]. These ROS scavenge NO thereby preventing vasodilation. Upregulation of Ca channels by oxy-Hb may increase the sensitivity of the cerebral vasculature to contractile stimuli and both prolong and potentiate vasoconstriction [18]. Furthermore, oxy-Hb also results in an inflammatory cascade in the walls of the cerebral vessels. Inflammation activates iNOS which, in contrast to its constitutively active counterparts, causes cellular damage by generation of NO in an oxidative environment which then reacts with free radicals to propagate ROS formation. The elevation of ROS levels by iNOS results in further local vascular inflammation. Activation of the inflammatory cascade is induced at the ictus of SAH and contributes to the subsequent development of CV. In an experimental SAH model in primates, infiltration of inflammatory cells into the walls of cerebral vessels was shown to be the highest, one week following SAH induction, which correlated with the peak severity of angiographic vasospasm [19].

Proteins which promote cell-cell interactions have been shown to be upregulated during the inflammatory response in order to facilitate the recruitment, adhesion, and transmigration of leukocytes [20]. Intercellular adhesion molecule-1 (ICAM-1) is a ligand for the receptor lymphocyte function-associated antigen-1 (LFA-1) which is ubiquitously expressed not only on all T-cells but also on other immune cells such as neutrophils, macrophages, and B-cells. The coupling of ICAM-1 to LFA-1 is a crucial initial step to the recruitment of inflammatory cells to the vessel wall. Increased expression of ICAM-1 has been demonstrated in endothelial cells following exposure of the adventitia to blood [21]. Aihara et al. [22] measured the level and evaluated the time course of cytokine and cell adhesion molecule gene expression following induction of SAH in canines. They determined that the peak expression of interleukin-1 (IL-1), IL-6, IL-8, and ICAM-1 was seven days after SAH which correlated with maximal arterial narrowing on angiography. These results implicate a prolonged inflammatory response in CV with a potential correlation between the magnitude of inflammation and the severity of vasoconstriction.

In addition to ICAM-1, other cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, have shown to be increased in the CSF of patients following aneurysm rupture [23]. Nissen et al. [24] studied the serum levels of multiple cell adhesion molecules in aneurysmal SAH patients with and without delayed ischemic neurological deficit (DIND) and did not find differences in the levels of ICAM-1, VCAM-1, platelet endothelial cell adhesion molecule-1 (PECAM-1), or E-selectin between the two cohorts. The levels of P-selectin and L-selectin were significantly higher and lower, respectively, in the patients who developed DIND. While there is clearly a link between inflammation and CV, it is clear that the use of CSF and serum biomarkers to assess this correlation is imperfect. Refinement of current approaches or development of new biomarker assays is necessary before these approaches can achieve widespread clinical applicability.

SMC contractility is not only mediated by SAH-induced variations in ET-1 and NO levels, but also by alteration in the electrochemical balance of ions such as Ca, sodium (Na), potassium (K), and chloride (Cl). SAH causes SMC depolarization by activation of Ca and Na channels and inactivation of K channels. Nimodipine, a Ca channel blocker (CCB), is the only pharmacologic agent which has been clinically proven to reduce delayed morbidity and mortality from aneurysmal SAH, although it does not reduce the incidence of angiographic CV [25–27]. The clinical benefit of endovascular administration of intra-arterial CCBs, such as verapamil, is currently equivocal [28]. The K channel activator cromakalim has been shown, in an *in vivo* rabbit SAH model, to ameliorate vasospasm [29]. Pathological alterations in ion channel physiology are crucial mechanisms underlying the molecular and clinical manifestations of CV. However, artificial manipulation of ion balance alone is inadequate to prevent or reverse the disease process.

Additional evidence suggests that SMC contractile tone and the mechanisms which regulate SMC contraction may change over the time course of CV [30]. This suggests that changes in SMC physiology may be induced by SAH. While the role of SMC phenotypic modulation in aneurysm formation, progression, and rupture has been studied, its role in the inflammation associated with CV is currently unknown [31]. A recent study by Kim et al. [32] identified a single nucleotide polymorphism in the gene encoding the NaCl cotransporter SLC12A3. Despite extensive research into the molecular biology and biochemistry of aneurysmal SAH-induced CV, the mechanisms underlying its pathogenesis remain incompletely understood.

3. Pathogenesis of Aneurysmal Subarachnoid Hemorrhage-Induced Delayed Cerebral Ischemia Unrelated to Cerebral Vasospasm

DCI unrelated to CV is becoming increasingly recognized as a significant contributor to delayed morbidity in aneurysmal SAH patients. These non-CV sources of neurological dysfunction may provide an explanation for the continuing failures of clinical trials using pharmacological inhibitors which target CV [33, 34]. In addition to cerebral ischemia secondary to lack of blood supply in the setting of CV-induced vasoconstriction, global DCI occurs following SAH secondary to activation of proapoptotic pathways [35]. The initiation of proapoptotic mechanisms likely occurs with the acute brain injury which accompanies the ictus of SAH. The rapid increase in intracranial pressure cannot be compensated for by higher levels of cerebral blood flow thereby resulting in a decrease in cerebral perfusion pressure (CPP). This sudden drop in CPP activates the stress response transcriptional regulatory protein hypoxia-inducible factor 1α (HIF-1α) [36]. HIF-1α increases the production of BCL2/adenovirus E1B 19 kDa protein-interacting protein (BNIP3) which promotes apoptosis by releasing cytochrome c from the mitochondria. This subsequently activates downstream caspases and by sequestering the antiapoptotic protein Bcl-2.

In addition to modulation of apoptotic pathways, SAH also causes disruption of the blood-brain barrier (BBB) by activation of matrix metalloproteinases (MMPs) which degrade the vascular basement membrane [37, 38]. Park et al. [39] observed increased BBB permeability, increased cerebral edema, and apoptosis of endothelial cells and hippocampal and cortical neurons after SAH induction in rats. Administration of a pan-caspase inhibitor reduced BBB permeability, prevented development of cerebral edema, and improved neurological outcome. The integrity of the BBB is not only important in limiting the accumulation of cerebral edema, but it is also linked to the prevention of proinflammatory signals and neuronal apoptosis [40]. Another cerebrovascular alteration following SAH is the dysfunction of the microcirculation. Rather than vasoconstriction of large arteries, some studies have suggested that the narrowing of small parenchymal arterioles contributes to pathological alterations in regional cerebral blood flow and to the development of DCI [41, 42].

Microvascular platelet aggregation following SAH is another potential mechanism which may mediate DCI secondary to small vessel thrombosis and cortical and subcortical ischemia. Sehba et al. [43] detected microvascular platelet aggregation in SAH-induced rats by immunostaining for the glycoprotein IIb/IIIa (GPIIb/IIIa), the receptor on activated platelets responsible for mediating fibrin cross-linking. An autopsy study of 29 patients who died from aneurysm rupture identified a statistically significant correlation between the magnitude of microvascular thromboembolism burden, detected by immunostaining, and the histologic evidence of ischemia at autopsy as well as clinical evidence of DCI prior to death [44].

In addition to microcirculatory disease, widespread cortical depression may predispose SAH patients to DCI. Dreier et al. [45] performed electrocorticography on patients who were surgically treated for ruptured aneurysms and found spreading depolarizations in 72%. The electrocorticographic measurement of recurrent spreading depolarizations had 86% and 100% positive and negative predictive values, respectively, for the development of delayed ischemic neurological deficits. The authors proposed that repeated spreading depolarizations with prolonged depressions could predict the subsequent occurrence of DCI. It is likely that many of

the aforementioned pathological mechanisms are interrelated with the development, propagation, and worsening of CV [46]. However, past clinical outcomes from aneurysmal SAH studies have taught us that the reversal of angiographic CV alone is inadequate to ameliorate the delayed morbidity and mortality associated with the rupture of an intracranial aneurysm.

4. Role of Estrogen in the Treatment of Cerebral Vasospasm

4.1. Estrogen Physiology. E2 is the most potent endogenous estrogen. Like other steroid hormones, E2 is derived from cholesterol. Cholesterol is initially converted to the intermediate progesterone products, pregnenolone and 17α-hydroxypregnenolone, which are then converted to the androgen intermediates, dehydroepiandrosterone, androstenediol, androstenedione, and testosterone. The androgen testosterone is then converted by the final enzyme in the synthetic pathway, aromatase, into E2. In a parallel pathway, androstenedione is converted by aromatase into estrone (E1) which, *in vivo*, is interconvertible with E2 [47]. E2 passes through the cell membrane to bind to the two isoforms of the estrogen receptor (ER), ERα and ERβ, in the cytoplasm. The E2-ER complex then enters the cell nucleus to regulate the transcription of multiple genes [48]. Pharmacologic blockade of the physiologic effects of E2 targets the synthetic pathway or the receptor. E2 synthesis is decreased with gonadotropin-releasing hormone (GnRH) agonists (e.g., leuprolide, goserelin) and aromatase inhibitors (e.g., anastrozole, exemestane). Antagonists of the ER are more properly termed selective estrogen receptor modulators (SERM) since, rather than being pure antagonists, they are simultaneously partial agonists as well as antagonists [49]. The action of SERMs is tissue specific and varies depending on the relative ratio of coactivator to corepressors and on the conformation of the ER.

4.2. Effect of Estrogen on the Vasculature during Cerebral Vasospasm. E2 is a powerful vasodilator with the potential to prevent or reverse the vasoconstriction which occurs in CV. *In vitro* studies have demonstrated that E2 binding to ERα results in activation of eNOS through MAPK-dependent pathways [50]. *In vivo* evidence from continuous E2 treatment of SAH-induced animals showed attenuation of CV, decreased SAH-induced iNOS expression, and normal eNOS expression [51]. This implicates a dual role of E2 in the prevention of SAH-induced iNOS upregulation and the maintenance of normal eNOS activity (which is typically suppressed in the setting of SAH). Mechanistic data from *in vitro* studies by Zancan et al. [52] demonstrated abrogation of cytokine-induced iNOS upregulation by E2 treatment in cultured rat aortic SMCs. Blockade of E2 signaling with an ERα antagonist resulted in the absence of E2 modulation of iNOS expression.

Shih et al. [53] treated SAH-induced rats with E2 and a nonselective ER antagonist and found that E2 prevented post-SAH elevation of iNOS levels and CV in an ER-dependent mechanism. The study also examined the levels of p65, a subunit of nuclear factor κ light chain enhancer of activated B cells (NFκB) and identified increased association of p65 and ER following administration of E2. The nuclear translocation of p65 was unaffected by E2 treatment. Therefore, a potential mechanism of E2-mediated vasodilation is the cytoplasmic sequestration of the transcription factor NFκB by ER which prevents the NFκB-dependent upregulation of iNOS instigated by aneurysmal SAH. Studies in extracranial vasculature have suggested that E2 may potentiate the effect of MMPs on the modulation of ET-mediated vasoconstriction [54]. Additional *in vivo* data from an experimental SAH model in rats demonstrated significantly decreased levels of ET-1 production in the cohort treated with E2 [55]. The ET-1 levels of the SAH animals treated with E2 were not significantly different from ET-1 levels of control animals. The mechanisms by which E2 mediates vasodilation are depicted in Figure 1.

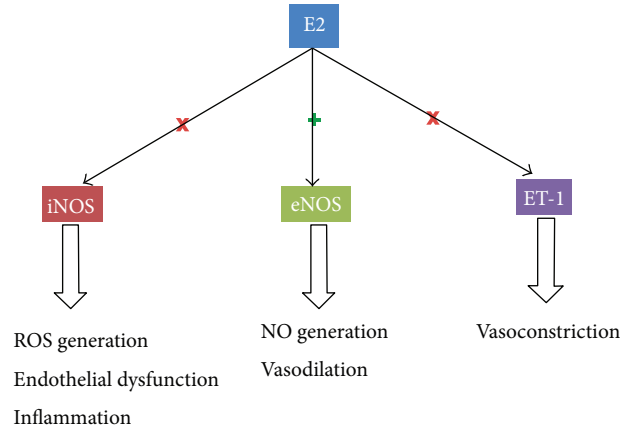

Figure 1: Pathways of E2-mediated facilitation of vasodilation and inhibition of vasoconstriction. E2: 17β-estradiol, eNOS: endothelial nitric oxide synthase, ET-1: endothelin-1, iNOS: inducible nitric oxide synthase, NO: nitric oxide, and ROS: reactive oxygen species.

5. Neuroprotective Mechanisms of Estrogen in the Setting of Aneurysmal Subarachnoid Hemorrhage

Evidence suggests that E2 may have neuroprotective properties [56]. E2 appears to diminish the risk of ischemic stroke and neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [57]. Not all neuroprotection afforded by E2 is mediated by ER. Early studies found E2 to have antioxidant effects, via scavenging of ROS, which were unaffected by tamoxifen treatment [58]. Lee et al. [59] showed that E2 increased expression of the antioxidant thioredoxin (Trx) in a cGMP-dependent manner. Trx was demonstrated, in the same study, to abrogate lipid peroxidation, caspase-3 activation, and apoptosis in response to oxidative stress. Srivastava et al. [60] found that E2 decreased expression of the critical proinflammatory cytokine tumor necrosis factor α (TNFα) by causing reduced activity of c-Jun N-terminal kinase (JNK). Depression of JNK results in decreased phosphorylation of its

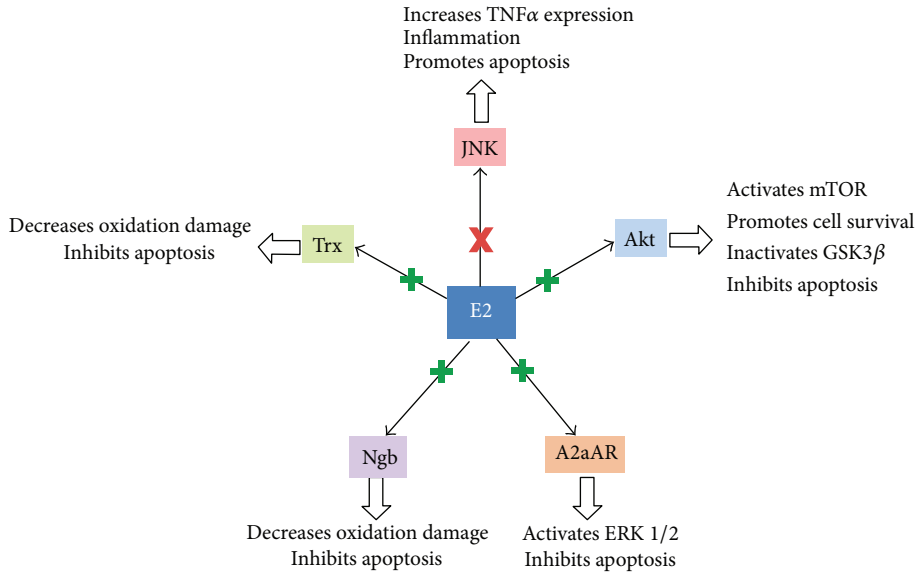

Figure 2: Pathways of E2-mediated neuroprotection. Akt: protein kinase B, E2: 17β-estradiol, ERK1/2: extracellular signal-regulated kinases 1 and 2, GSK3β: glycogen synthase kinase 3β, JNK: c-Jun N-terminal kinase, mTOR: mammalian target of rapamycin, Ngb: neuroglobin, TNFα: tumor necrosis factor α, and Trx: thioredoxin.

downstream targets which heterodimerize to form the transcription factor activator protein-1 (AP-1). AP-1 transactivates TNFα by binding to its promoter region. Thus, E2-mediated disruption of AP-1 formation decreased transcription of TNFα. Xing et al. [61] subsequently demonstrated that the anti-inflammatory actions of E2 were dependent on the ERβ receptor isoform. In addition to decreasing TNFα expression, E2 binding to ERβ also hindered neutrophil chemotaxis by decreasing expression of P-selectin, ICAM-1, VCAM-1, monocyte chemoattractant protein-1 (MCP-1), and cytokine-induced neutrophils chemoattractant-2β (CINC-2β).

Neuroglobin (Ngb) is a protein which regulates neuronal oxygen homeostasis by binding to oxygen with a higher affinity than hemoglobin [62]. While the precise mechanism of Ngb has yet to be delineated, it likely contributes to the protection of the brain from oxidative damage by ROS. De Marinis et al. [63] found that *in vitro* treatment of mouse hippocampal neurons with E2 resulted in a threefold increase in Ngb levels which was mediated by the ERβ receptor. The ERβ-mediated upregulation of Ngb expression was dependent on the p38 class of MAPKs. Additionally, E2 afforded protection against apoptosis induced by hydrogen peroxide. This protective effect was abrogated in Ngb-silenced cells. Hota et al. [64] provided *in vivo* data to support the antiapoptotic role of Ngb during the neuronal stress response to hypoxic stimuli. Ngb was shown to stabilize the transcription factors HIF-1α and nuclear factor erythroid 2-related factor 2 (Nrf2) and prevent mitochondrial release of the caspase-activating protein cytochrome c.

Recent *in vivo* evidence presented by Kao et al. [65] implicates the Akt signaling pathway in E2-mediated neuroprotection. Akt, otherwise termed protein kinase B (PKB), is downstream from phosphoinositide 3-kinase (PI3K) and upstream from the kinase known as mammalian target of rapamycin (mTOR). This complex signaling pathway involving the three kinases PI3K, Akt, and mTOR, integrates multiple inputs in order to promote cell growth and proliferation [66]. Downstream inactivation of glycogen synthase kinase 3β (GSK3β) by Akt is known to inhibit apoptosis [67]. Activation of ERα by E2 was shown to inhibit apoptosis in the dentate gyrus of rats afflicted with SAH by depressing the activity of the proapoptotic enzyme caspase-3 and by preventing the SAH-induced decrease in signaling through Akt [65]. Prior studies potentiating signaling through Akt-GSK3β pathway by other mechanisms have also shown attenuation of neuronal cell death in the setting of SAH [68, 69]. Furthermore, E2 has been found to exert antiapoptotic effects through upregulation of adenosine A2a receptor (A2aAR) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) expression [70]. The activation of A2aAR and ERK1/2 results in inhibition of downstream apoptotic signaling pathways. The mechanisms by which E2 mediates neuroprotection are depicted in Figure 2. Table 1 summarizes the molecular mechanisms underlying E2-mediated vasodilation and neuroprotection.

6. Limitations of Current Studies and Future Directions

Estrogen treatment is associated with multiple potential adverse effects which may dampen the enthusiasm for its use in the treatment of aneurysmal SAH patients. These risks include malignancies of the breast, endometrium, and ovaries, dysmenorrhea, gastrointestinal dysfunction, dyslipidemia, venous thrombosis, pulmonary embolism, myocardial infarction, and stroke [71–73]. Due to the relatively short clinical time course during which estrogen would be administered to treat CV and DCI (i.e., approximately two

Table 1: Summary of vasodilatory and neuroprotective mechanisms regulated by 17β-estradiol (E2).

Mediator	Physiology
Vasodilatory mechanisms regulated by E2	
Endothelial nitric oxide synthase (eNOS)	Constitutively expressed isoform of NOS which generates the vasodilatory mediator NO. E2 activates eNOS and prevents the SAH-induced decrease of eNOS function via MAPK-dependent pathways.
Inducible nitric oxide synthase (iNOS)	Inducible isoform of NOS expressed in stress responses (e.g., SAH) which contributes to the generation of reactive oxygen species. E2 abrogates SAH-induced iNOS expression by sequestering NFκB, a transcriptional activator of iNOS.
Endothelin-1 (ET-1)	The most potent endogenous mediator of vasoconstriction. E2 decreases ET-1 production.
Neuroprotective mechanisms regulated by E2	
Thioredoxin (Trx)	Antioxidant enzyme which reduces oxidized proteins and diminishes stress-induced proapoptotic signaling. E2 increases Trx expression.
c-Jun N-terminal kinase (JNK)	JNK phosphorylates downstream proteins which heterodimerize to form AP-1, a transcriptional activator of TNFα. E2 decreases JNK activity.
Neuroglobin (Ngb)	Globin protein which binds to oxygen with a greater affinity than hemoglobin thereby regulating oxygen homeostasis in neurons. Ngb provides protection against ROS-induced oxidative damage and prevents apoptosis by stabilizing the transcription factors HIF-1α and Nrf2 and by inhibiting cytochrome c release from the mitochondria. E2 increases Ngb expression.
Protein kinase B (Akt)	Akt activates mTOR which promotes cell survival and inactivates GSK3β which promotes apoptosis. E2 prevents SAH-induced suppression of Akt.
Adenosine A2a receptor (A2aAR)	G-protein couple receptor which inhibits proapoptotic signaling pathways partially through activation of ERK 1/2. E2 increases expression of A2aAR and ERK 1/2.

weeks), we believe the increased risk of estrogen-related cancers would be negligible. However, the potential systemic adverse effects of estrogen on patients, especially those with preexisting medical comorbidities, may be significant. It is possible that thromboembolic complications associated with estrogen therapy may offset any benefits afforded by its vasodilatory or neuroprotective mechanisms. While estrogen has been demonstrated to diminish CV and DCI in animal SAH models, it has yet to be tested in human trials for the treatment of patients with ruptured aneurysms. Therefore, the clinical safety profile and efficacy of estrogen have yet to be determined. Future studies are necessary to establish a dose-response relationship for estrogen in order to initiate early phase clinical trials.

7. Conclusions

Estrogen, specifically E2, possesses powerful vasodilatory, anti-inflammatory, and neuroprotective properties. Its current use for the treatment of CV remains limited to *in vivo* animal models of experimental SAH. It appears that the significant majority of E2-mediated neuroprotection occurs via ERα- and ERβ-dependent mechanisms. The contribution of ER-independent mechanisms of E2 neuroprotection is relatively small. Therefore, successful pharmacologic modulation of ERs may provide a potential target of future clinical studies of E2 for the treatment of CV and DCI following aneurysmal SAH.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Clinical Study

Description of the Vasospasm Phenomena following Perimesencephalic Nonaneurysmal Subarachnoid Hemorrhage

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Background. Perimesencephalic nonaneurysmal subarachnoid hemorrhage (PM-NASAH) is characterized by a benign course compared with aneurysmal SAH. While vasospasm (VS) after aneurysmal SAH is considered responsible for serious complications, VS post-PM-NASAH is not well documented. Our purpose was to characterize the incidence and course of VS among 63 patients—one of the largest databases of PM-NASAH patients with documented blood flow velocities in the literature. *Methods*. Data from 63 patients that were admitted with PM-NASAH from 2000 to 2012 and underwent transcranial Doppler tests to assess cranial vessel flow velocity was analyzed. *Results*. On average, the maximal flow velocity was measured on the 7th day after hemorrhage. Higher risk for VS was associated with younger age, female sex, and higher Hunt and Hess scores, a lower risk for patients treated with statins ($P < 0.05$). Using velocity thresholds for diagnosis of VS, 49.2% showed evidence of VS. This is the first description of blood flow velocities in PM-NASAH. VS average onset was on the 4th day, average cessation on day 15 after hemorrhage. No patients showed clinical manifestation of VS. *Conclusions*. VS post-PM-NASAH is not as rare as previously believed. However, its lack of clinical significance raises questions regarding the need for diagnosis and may suggest a less intensive treatment protocol.

1. Introduction

Nontraumatic subarachnoid hemorrhage (SAH), comprising twenty percent of all SAH [1], has an estimated annual incidence of 6 cases per 100,000 persons in the United States [2]. Spontaneous SAH is most often associated with ruptured intracranial aneurysms (70–80%) and less frequently with arteriovenous malformations (4%) [1]. However, in approximately 15% of all patients presenting with spontaneous SAH, the cause of the bleeding remains unknown even after detailed imaging studies [3, 4].

In two-thirds of SAH cases with normal imaging studies, the blood accumulates predominantly around the midbrain [5]. Such bleeding patterns were first described by van Gijn and colleagues in 1985 [6] and were termed perimesencephalic nonaneurysmal subarachnoid hemorrhage (PM-NASAH). The distinct radiographic pattern of PM-NASAH comprises of a hemorrhage located anterior to the midbrain or the pons, with or without an extension of blood around the brainstem, the suprasellar cistern, or the proximal sylvian fissures [2].

The etiology of PM-NASAH has not yet been determined [5]. Given its typically focal site and low volume of bleeding, it is likely that PM-NASAH represents the rupture of low-flow entities, such as a local venous or capillary structure, a perforating artery, a low-flow vascular malformation, or a capillary telangiectasia [7]. Compared with aneurysmal SAH (ASAH), PM-NASAH is associated with a better outcome, but recurrent bleeding and delayed cerebral ischemia may occur [8].

Although VS following ASAH is well documented, VS in the context of PM-NASAH is believed to be rare, with few reports present in the literature [7]. The pattern of VS in PM-NASAH is typically confined to the posterior circulation, although diffuse VS has been described [9]. Within a small subgroup of PM-NASAH patients who develop angiographic evidence of VS, only a small number of individuals become clinically symptomatic [7]. Rinkel and colleagues reported on a series of 65 patients with PM-NASAH, in which only 3%

had angiographic evidence of VS [10]. In a smaller series of 20 patients, van Calenbergh and colleagues noted that although 20% of patients had angiographic VS, only one patient has developed neurologic deficit [11].

While PM-NASAH is considered as a subtype of angiographically negative SAH (AN-SAH), it is important to distinguish the clinical significance of VS in these groups. This difference is illustrated by Gross et al., in which among 77 patients who were evaluated with delayed angiography, 26% suffered from VS, 4% developed delayed infarcts, and 4% deteriorated due to delayed cerebral ischemia [12]. A diffuse hemorrhage pattern and a higher Hunt and Hess (H&H) grade were found to be risk factors for these complications. Hui et al. reviewed 94 patients with AN-SAH and found that the subgroup of patients with PM-NASAH was associated with a better outcome compared with the rest of the subgroups, with decreased risk for vasospasm and hydrocephalus [13]. Andaluz and Zuccarello found that compared with PM-NASAH, other types of AN-SAH were associated with significantly longer hospitalization and intensive care unit stay, greater complication rates, and worse outcomes [14].

The aims of the current study were to accurately assess the flow velocities in PM-NASAH and to describe, for the first time, the phenomenon of VS following PM-NASAH in the largest cohort of PM-NASAH patients with documented blood velocities established up to date.

Another aim was also to define a workup algorithm for VS, which provides the early diagnosis of clinically significant VS, and by thus decrease morbidity without prolongation of hospital stay.

2. Methods

2.1. Study Design. We used a retrospective cohort design in order to assess the risk factors for VS and its clinical outcomes after PM-NASAH.

2.2. Setting. We retrieved data from a prospective database that consisted of demographic data, clinical data, and transcranial Doppler (TCD) parameters for patients with SAH, admitted to the department of Neurosurgery at Sheba Medical Center, Tel-Hashomer, Israel, from January 1, 2000, to October 31, 2012. Data was compiled in accordance with the local IRB—Helsinki committee number 8199-10.

2.3. Participants. 63 patients met the following inclusion criteria: (1) a computed tomography (CT) scan performed on admission showed a perimesencephalic (PM) pattern of hemorrhage, (2) without an aneurysm as a cause of the bleeding, on angiographic analysis. A PM pattern of hemorrhage was defined as (1) an evidence of bleeding in the PM, interpeduncular and prepontine cisterns, (2) without involvement of the brain parenchyma, the ventricular system, or the sylvian fissure, and (3) without indication of an aneurysm or other source of bleeding. A mild extension of the bleeding into the proximal sylvian fissure was still considered as a PM bleeding and was named PM Plus (PM+). Pregnant women, patients under the age of 18 or over the age of 80, and patients lacking judgment were excluded from this study.

2.4. Variables. For each patient, we recorded demographic data, smoking habits, and concurrent diseases such as hypertension or diabetes. We collected the following data for each hemorrhage: date of onset, symptoms and signs at the time of admission, possible triggers, and clinical grading at the time of admission according to the H&H grading scale [15]. We also recorded the imaging modalities performed for each patient, the treatment received, and the clinical outcome.

Informed consent was waived by the local IRB for a retrospective study.

2.5. Data Sources and Measurement. Data regarding demographics, smoking habits, and concurrent diseases was delivered by the patients. Data regarding hemorrhage details (onset, symptoms and signs, and H&H score) was recorded by the patients' physicians.

2.5.1. Measuring Vasospasm. TCD is based on the principle that, given a constant cerebral blood flow, the flow velocity is inversely proportional to the vessel lumen cross section area. TCD is noninvasive, it is repeatable and inexpensive, and it can be performed at the patient's bedside. The most recent 2012 American Heart Association guidelines for the management of ASAH state that TCD is a reasonable method to monitor the development of arterial VS [16].

In the current study, a single trained operator (Bela Brule) performed all TCD examinations for all patients. TCD was performed daily as a part of routine follow-up examinations of all patients with spontaneous SAH (aneurysmal and nonaneurysmal).

Angiography (DSA or CTA) was performed on admission to rule out aneurysm or other vascular malformation that could be the cause of SAH and was repeated mostly in cases of PM+ type of SAH.

Flow velocities were measured in the distal portion of the extracranial internal carotid artery (ICA), proximal and distal middle cerebral artery (MCA), anterior cerebral artery (ACA), posterior cerebral artery (PCA) and vertebral artery (VA) bilaterally, and proximal and distal basilar artery (BA).

We used specific threshold criteria to define arterial VS, based on previously published reports [17–22]. The criteria included a mean flow velocity (MFV) in the intracranial ICA, MCA, and ACA greater than 120 cm/sec [18, 19]; a MFV in the VA greater than 80 cm/sec [20]; a MFV in the BA greater than 85 cm/sec [20]; a MFV in the PCA greater than 110 cm/sec [20, 21]; an extra-/intracranial ratio 2 for the carotid watershed (MCA, ACA, or intracranial ICA) greater than 3 [18]; and a proximal/distal ratio for the vertebrobasilar (VB) system greater than 2 [22].

TCD was performed daily to document velocities and to detect VS. VS and its course was observed and documented based on the following criteria. The day of VS onset and remission, the day of maximal velocity, the measured velocities, and the clinical outcome and treatment. Treatment was administered according to the treating physician (either Sagi Harnof or Moshe Hadani) and adhered to the protocol for active treatment of clinical VS only. Close monitoring was performed for TCD based VS or documented increased velocities.

Table 1: Clinical features of study group.

Variable	Patients
Total patients number	**63 (100%)**
Sex	
Male	34 (54%)
Female	29 (46%)
Hunt and Hess score on admission	
I	48 (76.2%)
II	12 (19%)
III	2 (3.2%)
IV	1 (1.6%)
Age	
Minimal	24
Maximal	75
Average	52.44
Vascular risk factors	
None	33 (52.4%)
Hypertension	19 (30.2%)
Diabetes Mellitus	9 (14.3%)
Other	2 (3.2%)
TCD performed	
Total	**241**
Per patient	
Minimal number of exams	2
Maximal number of exams	8
Average number of exams	3.76

2.6. Bias. Due to its retrospective design the study is inherently influenced by selection bias and information bias. No other bias as far as we are aware have influenced the results.

2.7. Study Size. The number of cases in the area during the study period determined the sample size.

2.8. Quantitative Variables. As mentioned above, the clinical significance of VS in PM-NASAH has yet to be determined. As such, we have chosen to describe VS using the accepted thresholds as well as by measuring the flow velocities of the intracranial arteries for all patients and identifying correlations between the velocities and patient characteristics.

2.9. Statistical Methods. Quantitative and continuous variables were described using sample size, mean, median, minimal and maximal values, and dispersion variables. Categorical and discrete variables were described using group size and observed and relative frequencies. Statistical analysis was performed with SPSS 20 for Windows (SPSS, Chicago, IL). The Chi-square test, Pearson's correlation, and Student's t-test were used. A P value <0.05 was considered significant.

3. Results

3.1. Participants. Among all patients admitted to our department with PM-NASAH during the study period, 63 patients met the inclusion criteria and were included in this study.

Table 2: VS defined by velocity thresholds—distribution by arteries involved.

Variable	Patients
Artery in which VS was detected	
Distal BA	24 (77.4%)
MCA	12 (38.7%)
Proximal BA	5 (16.1%)
ACA	4 (12.9%)
PCA	3 (9.6%)
VA	2 (6.4%)
ICA	0
Number of arteries involved per patient	
1	19 (61.4%)
2	5 (16.1%)
3	5 (16.1%)
4	1 (3.2%)
5	1 (3.2%)
Total	**31 (100%)**
Average	1.71

ICA: internal carotid artery; MCA: middle cerebral artery; ACA: anterior cerebral artery; PCA: posterior cerebral artery; VA: vertebral artery; BA: basilar artery; VS: vasospasm.

3.2. Descriptive Data. Among all 63 patients, 29 (46%) were females. The youngest patient was 24 years old and the oldest 75. The average age of our patients was 52.4. Hunt and Hess score on admission was 1 for 48 (76.2%) patients and higher for the rest (Table 1). Thirty-three (52.4%) of our patients were not reported to have vascular risk factors such as hypertension or diabetes mellitus. A total of 241 TCDs were performed for all patients, with an average of 3.76 TCDs performed per patient.

3.3. Outcome Data. As mentioned previously, we used velocity thresholds to diagnose possible VS. Using these thresholds, 31 of the 63 patients (49.2%) exhibited VS. Among these patients, more than three-quarters (24 cases—77.4%) had VS documented in the distal BA; other arteries in which VS was observed were the MCA (38.7%), proximal BA (16.1%), ACA (12.9%), PCA (9.67%), and VA (6.4%) (Table 2).

3.4. Main Results

3.4.1. Vasospasm Defined by Velocity Thresholds. The onset of VS occurred between the 2nd and the 8th day after hemorrhage, with the average being on the 4th day. The spasm ended between the 9th and 25th day after hemorrhage, with the average being the 15th day. Although the maximal velocity for all patients was measured between the 2nd and 16th, with the average being the 7th day after hemorrhage, in the VS patient group, the maximal velocity was measured between the 5th and the 16th day after hemorrhage, with the average being the 8th day after hemorrhage.

We examined the differences between patients in the VS and non-VS groups. Using Student's t-test analysis to examine the relationship between age and vasospasm, we found that younger patients were more susceptible to VS (6.84 years younger on average, $P = 0.008$). No difference was found for sex, H&H score, vascular risk factors, smoking, and the season of the year in which the hemorrhagic event took place.

3.5. Other Analyses

3.5.1. Flow Velocities in PM-NASAH. We examined the correlations between the mean flow velocities, patients, and SAH characteristics. Using Pearson's correlation, we found a negative correlation between the mean flow velocity in the distal BA and distal BA/VA and patient age ($P = 0.03$ and 0.02, resp.). This finding correlates with the results mentioned above, showing a possible higher risk for VS in younger patients. When comparing flow velocities and patient sex using Student's t-test analysis, we found that the mean flow velocities among women were higher, when measured on the ICA, VA, and proximal BA ($P = 0.001$, 0.014, and 0.001, resp.). Correlating the flow velocities with the H&H score using Student's t-test analysis, we found a higher mean flow velocity in the VA artery in the patients with H&H score of 2, 3, and 4 at admission, compared with patients with H&H score of 1 ($P = 0.027$).

We examined the relationship between statins treatment during hospitalization and VS and found a lower mean flow velocity in the ICA, ACA, VA, proximal BA, and distal BA arteries in patients treated with statins versus patients untreated with statins ($P = 0.002$, 0.035, 0.007, 0.008, and 0.013, resp.). No significant correlations were found between flow velocities and the following variables: smoking, season of the year, treatment with nimodipine during hospitalization, or other vascular risk factors. Overall, we found a significantly higher probability for VS with younger age, female sex, and a higher H&H score at the time of admission. Our findings suggest significantly lower risk for patients treated with statins. The minimum, maximum, and average flow velocities measurements in the different intracranial arteries are summarized in Table 3.

Figures 1, 2, and 3 summarize the flow velocity distributions. The day of maximal velocity was between the 2nd and the 16th day after the initiation of the hemorrhage, with the average being the 7th day.

3.5.2. Clinical Impact of Vasospasm. Symptomatic patients were categorized into two groups. The first group of patients exhibited symptoms that were recorded upon initial examination and were usually related to cranial nerves (CN) involvement (6 patients had CN involvement; CNs 3, 6, 7, or 8 were involved). The second group of patients had presented with a worsening headache that developed during hospitalization and responded to medical treatment including drainage of a cerebrospinal fluid (CSF). Nevertheless, none of the patients demonstrated symptoms that could be attributed to arterial VS, and none had manifestation of VS that could be tracked clinically.

Table 3: Summary of the minimum, maximum, and average flow velocities in the intracranial arteries.

Artery	Minimum (cm/sec)	Maximum (cm/sec)	Mean (cm/sec)	Std. deviation (cm/sec)
ICA	21	52	33.59	6.95
MCA	34	184	85.08	36.56
MCA/ICA	1.06	6.75	2.62	1.25
ACA	25	146	68.32	28.66
PCA	24	127	53.33	20.66
VA	19	90	42.25	15.23
Proximal BA	18	116	49.63	22.78
Distal BA	18	153	71.67	36.50
Distal BA/VA	0.59	5.05	1.73	0.83

ICA: internal carotid artery; MCA: middle cerebral artery; ACA: anterior cerebral artery; PCA: posterior cerebral artery; VA: vertebral artery; BA: basilar artery.

Table 4: Vasospasm after PM-NASAH versus ASAH.

	PM-NASAH VS in our series	ASAH VS in the literature
Incidence	49.2%	70% [23]
Clinical significance	None	Ischemic neurological symptoms in 50% of large artery VS [16]
Spasm onset*	4.9 d	3 d [23]
Day of max. velocity*	7.03 d	6–8 d [23]
Spasm resolution*	15.5 d	2-3 weeks [23]

*Average number of days/weeks after hemorrhage.

4. Discussion

4.1. Key Results. The current study characterizes, for the first time, the phenomena of VS following PM-NASAH, in one of the largest cohorts of patients with PM-NASAH described in the literature. We described the VS phenomenon in PM-NASAH as well as the arterial flow velocities among our patients. We examined correlations between these variables, patient characteristics, and SAH characteristics. We found possible risk factors and showed the possible benefit from treatment with statins.

4.2. Limitations. This study presents a historical cohort group of patients diagnosed and treated in retrospect, without the guiding hand in the data collection during the hospital stay. In addition, the study lacks long-term followup for patients, referring to the clinical implications of the described objective findings.

4.3. Interpretation. Our series shows that VS in PM-NASAH is different from VS after ASAH in several aspects (Table 4). Most notably, VS after PM-NASAH is less common than after ASAH, and its clinical significance is negligible in comparison to ASAH VS. In our series, 24 of all 63 patients (38%) had VS detected by TCD examination in the distal BA; however, the locations of the VS or the number of arteries

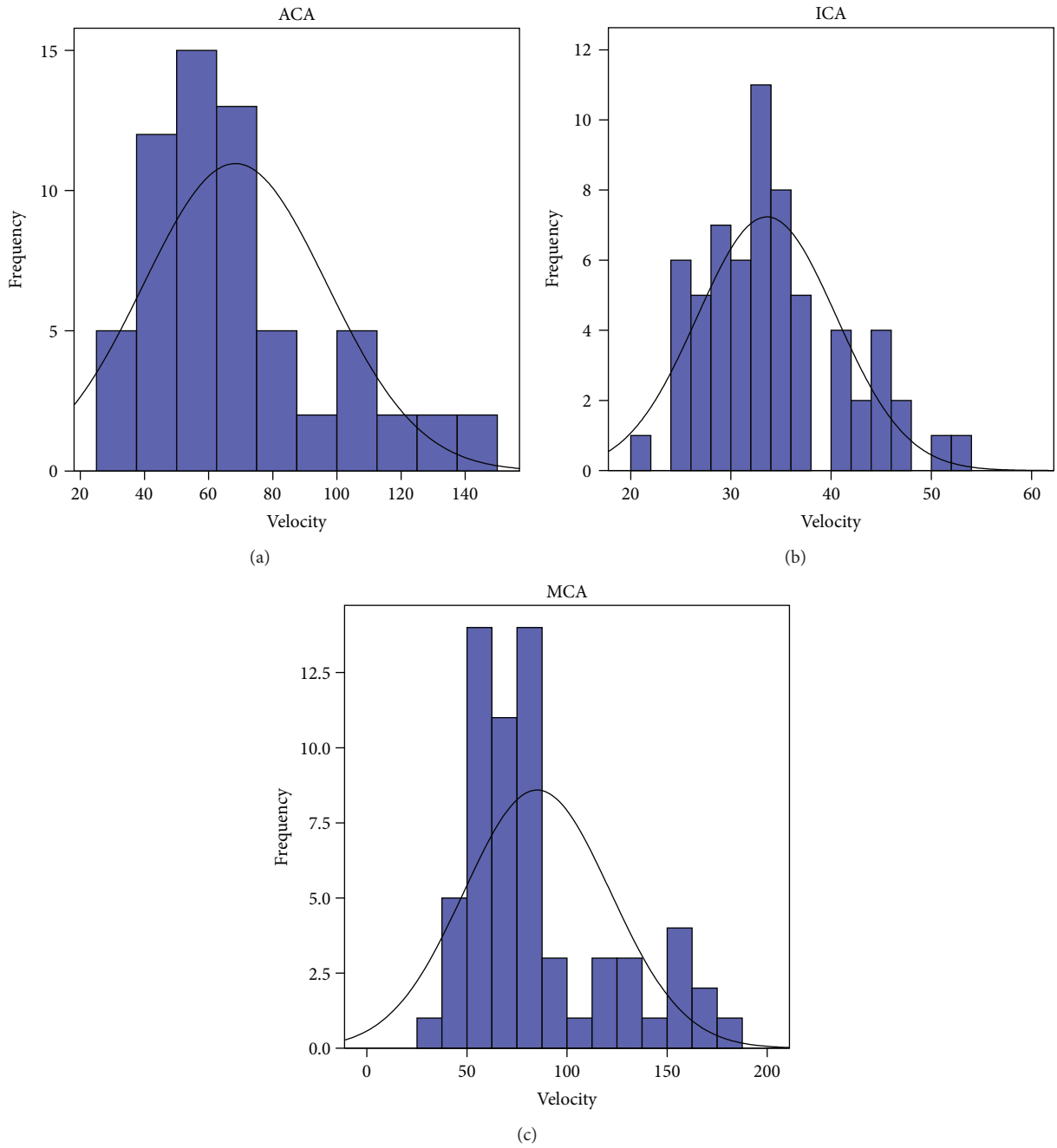

Figure 1: Flow velocity distributions in the anterior circulation (cm/sec). (MCA: middle cerebral artery; ICA: internal carotid artery; ACA: anterior cerebral artery.)

involved had no clinical impact. The onset day, maximal spasm day, and duration are similar to those in ASAH. These details emphasize the importance of establishing a protocol for monitoring and treating PM-NASAH.

4.4. Generalisability. The lack of clinical significance of VS in the PM-NASAH patients in our series resembles to the results of other studies. As such, we suggest that using a velocity threshold for VS in PM-NASAH has little significance. We strongly recommend clinical observation and clinically-based decision making regarding VS in the setting of PM-NASAH. The extremely low incidence of neurological deficit subsequent to VS should be taken under consideration when handling and observing PM-NASAH patients. Early discharge of these patients, based on the belief that ischemic symptoms will not occur, may be inadequate, since the exact incidence of PM-NASAH VS is still unknown and requires further investigation. We concur with the results of other studies [24] that early discharge of these patients may be suitable if adequate postdischarge observation is available from

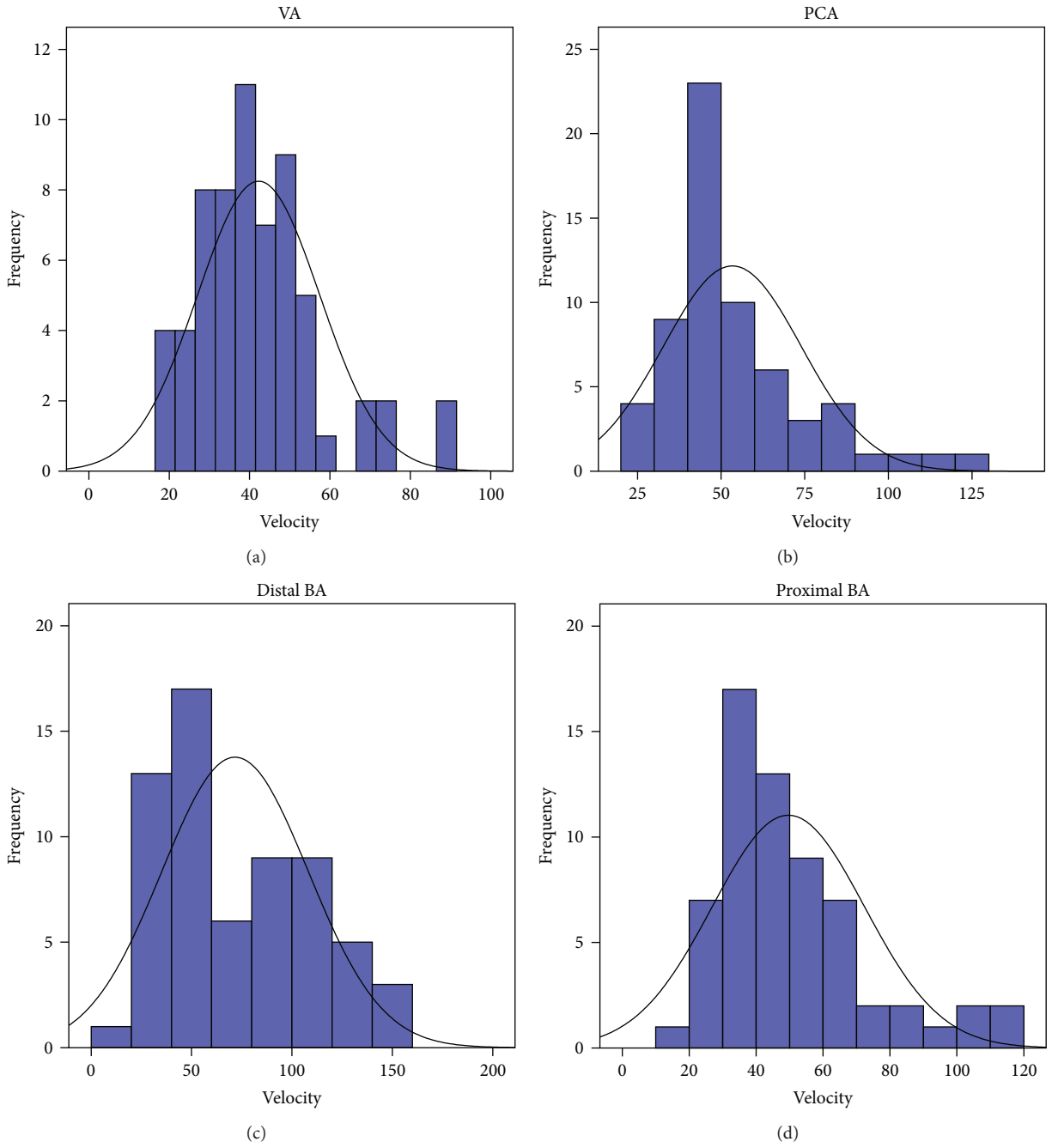

Figure 2: Flow velocity distributions in the posterior circulation (cm/sec). (PCA: posterior cerebral artery; VA: vertebral artery; BA: basilar artery.)

carefully trained observers. The proposed follow-up protocol for the patients with PM-NASAH includes baseline complete TCD examination within 24 hours after patient's admission and repeated TCD examinations only in cases of clinical symptoms, suggestive of vasospasm-related complications.

Angiography (DSA or CTA) should be performed on admission to rule out aneurysmal origin of SAH. Repeated angiographic examinations may be needed primarily in cases of PM+ for the same purpose.

5. Conclusion

To date, PM-NASAH VS is rarely cited in the literature. This complication is not as rare as previously thought, with almost 50% of patients with PM-NASAH examined in this series having imaging evidence of VS. However, the lack of its clinical significance raises questions regarding the need for diagnosis using the flow velocity threshold, as used in ASAH. The benign course of VS in the context of PM-NASAH may

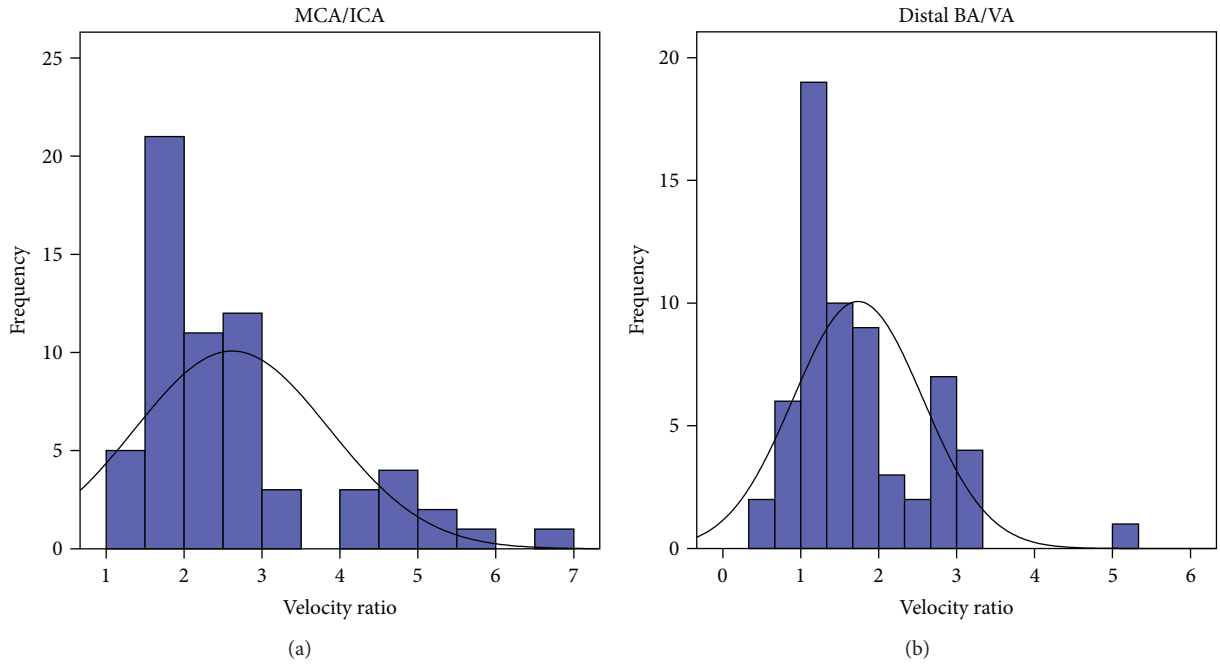

Figure 3: Flow velocity ratios. (MCA: middle cerebral artery; ICA: internal carotid artery; VA: vertebral artery; BA: basilar artery.)

suggest the use of a less intensive treatment protocol in comparison to ASAH. Early discharge may be considered, if adequate observation out-patient clinic is available. Nevertheless, although not exhibited by our patients, rare but dangerous sequelae are possible, and further research is needed in order to gain a more comprehensive understanding of the risks of the apparent benign nature of this condition.

Disclosure

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