

# Matrix metalloproteinase 9 may be involved in contraction of vascular smooth muscle cells in an *in vitro* rat model of subarachnoid hemorrhage

BAOQI DANG<sup>1,2\*</sup>, HAITAO SHEN<sup>2\*</sup>, HAIYING LI<sup>2</sup>, MIN ZHU<sup>1</sup>, CHUNHUA GUO<sup>1</sup> and WEICHUN HE<sup>1,2</sup>

<sup>1</sup>Department of Neurosurgery, Zhangjiagang Hospital of Traditional Chinese Medicine, Nanjing University of Chinese Medicine, Suzhou, Jiangsu 215600; <sup>2</sup>Brain and Nerve Research Laboratory, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, P.R. China

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**Abstract.** Our previous study determined that prominent cerebral vasospasm (CVS) may occur in an *in vivo* model of subarachnoid hemorrhage (SAH) in rats. Matrix metalloproteinase 9 (MMP-9) expression levels in basilar arteries were upregulated in a similar manner to the development of CVS following SAH. To identify the changes that occur in the contractility of cerebrovascular smooth muscle cells and the expression levels of MMP-9 in an *in vitro* model of SAH, rat cerebrovascular smooth muscle cells were isolated, cultured, and then stimulated with hemolysate. Additionally, 2-[(4-phenoxypheylsulfonyl)methyl]thiirane (SB-3CT), a selective MMP-9 inhibitor, was used to determine the effect of MMP-9 on the contractility of cerebrovascular smooth muscle cells. Cerebrovascular smooth muscle cells were successfully isolated and cultured *in vitro*, and hemolysate stimulation enhanced their contractility and increased MMP-9 expression levels. The present study also revealed that pretreatment with SB-3CT decreased MMP-9 expression levels in cerebrovascular smooth muscle cells, and reduced their contractility upon hemolysate treatment. Therefore, the current study confirmed that MMP-9 is important for the enhancement of the contractility of cerebrovascular smooth muscle cells in an *in vitro* rat model of SAH.

## Introduction

The surgical and endovascular treatments of aneurysms and neurological intensive care have greatly improved. Therefore, the possibility of a hemorrhage following an aneurysm re-rupture is well-controlled. However, cerebral vasospasm (CVS) continues to be the primary reason for mortality and disability in patients with subarachnoid hemorrhage (SAH) (1). The pathogenesis of CVS remains to be fully elucidated. CVS may be due to various factors, including the increase of cerebral levels of NO, oxidative injury, platelet activation and aggregation, that enhance cerebral vasoconstriction and reduce cerebral vasodilation (2-5).

Matrix metalloproteinase 9 (MMP-9) is part of the gelatinase subfamily of MMPs and is expressed in various cells in the brain. Activated MMP-9 is responsible for the degradation of several proteins which constitute the extracellular matrix, including collagen types IV, V, VII and X, elastin, fibrillin, osteonectin and laminin. Therefore, MMP-9 is important for the pathogenesis of intracranial aneurysms (6-8). Our previous study determined that CVS may develop in rats following SAH (9). Additionally, MMP-9 expression levels in basilar arteries were upregulated with a time course similar to that of the development of CVS. This suggests that MMP-9 may be involved in the pathogenesis of CVS (9).

To the best of our knowledge, this is the first study that has aimed to examine the association between CVS and MMP-9. Therefore, the present study aimed to determine the underlying molecular mechanisms of CVS pathogenesis. The importance of MMP-9 for the contraction of cerebrovascular smooth muscle cells was also investigated.

## Materials and methods

**Isolation and culture of cerebrovascular smooth muscle cells.** Four adult male Sprague-Dawley rats weighing 300-350 g were sacrificed by decapitation in each treatment group. A total of 12 rats were used in the present study, purchased from the Animal Center of Chinese Academy of Sciences (Shanghai, China). All animals were housed at a constant temperature of 22°C, under a 12-h light/dark cycle (lights switched on at 6:00 a.m.)

**Correspondence to:** Dr Weichun He, Department of Neurosurgery, Zhangjiagang Hospital of Traditional Chinese Medicine, Nanjing University of Chinese Medicine, 77 South Changan Road, Suzhou, Jiangsu 215600, P.R. China  
E-mail: zhenjiangdbq@163.com

\*Contributed equally

**Abbreviations:** CVS, cerebral vasospasm; MMP-9, matrix metalloproteinase 9; DMEM/F12, Dulbecco's modified eagle medium/Nutrient Mixture F-12; ROS, reactive oxygen species

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with free access to food and water. All rats were placed under general anesthesia prior to fixation-perfusion and euthanasia procedures. All procedures were approved by the Institutional Animal Care Committee of the Zhangjiagang Hospital of Traditional Chinese Medicine (Suzhou, China) and were performed in accordance with the guidelines of the National Institutes of Health on the care and use of animals. Whole brains were dissected under sterile conditions and immediately placed in a culture dish with Dulbecco's modified Eagle's medium supplemented with Gibco<sup>®</sup> nutrient mixture F-12 (DMEM/F12), 100,000 U/l penicillin and 100,000 U/l streptomycin (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Basilar arteries were dissected out in a laminar flow hood and rinsed repeatedly with DMEM/F12 medium supplemented with 100,000 U/l penicillin and 100,000 U/l streptomycin. (Thermo Fisher Scientific, Inc.). Following excision of the adventitial layer, the vessels were sectioned into small segments of length ~0.2 mm. The tissue was digested in 1 ml 0.1% collagenase I solution (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 37°C with 5% CO<sub>2</sub> for 30 min until the tissue appeared swollen. In order to digest the tissue further, 1 ml 0.125% trypsin solution (Gibco; Thermo Fisher Scientific, Inc.) was added for an additional 10 min. The dispersed cells were collected and transferred to a centrifuge tube, and DMEM/F12 medium supplemented with 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) was added to terminate digestion. The cell suspension was subsequently centrifuged at 200 x g for 5 min, and the supernatant discarded. The cells were resuspended in DMEM/F12 medium with 20% FBS, seeded into a 60 mm culture dish and incubated at 37°C with 5% CO<sub>2</sub>. The medium was changed every 3 days. After 10 days, the culture had reached 80-90% confluence, following trypsin digestion the third passage was used for further experiments.

**Preparation of hemolysate.** Hemolysate was prepared using a freeze-thaw method previously described (10), although with several modifications, to lyse red blood cells. A heparinized sterile syringe was used to collect 1 ml blood from the rat tail artery. The blood was transferred to a sterile centrifuge tube, and then centrifuged at 2,500 x g for 15 min at 4°C. The serum was discarded, and the red blood cells were resuspended in sterile distilled water. The cells were frozen at -20°C for 20 min and then immediately transferred to a 39°C water bath. After the cells had completely thawed, they were centrifuged at 12,000 x g for 30 min at 4°C. The supernatant containing the hemolysate was collected. Hemolysate concentration was calculated as follows: Concentration = (hemolysate mass - distilled water mass) / hemolysate volume. All procedures were performed under sterile conditions.

**Treatment of cells.** Certain of these experiments included 2-[(4-phenoxyphenylsulfonyl)methyl]-thiirane (SB-3CT), a selective MMP-9 inhibitor purchased from Sigma-Aldrich; Merck Millipore. As shown in Fig. 1A, the cells were randomly assigned into three groups: i) The control group, where cerebrovascular smooth muscle cells were cultured under normal conditions; ii) the hemolysate treatment group, where the cells were treated with 1 mg/ml hemolysate for 24 h; and iii) the SB-3CT treatment group, where cells were

pretreated with 1  $\mu$ M SB-3CT for 6 h, and subsequently treated with 1 mg/ml hemolysate for a further 24 h.

**Western blot analysis.** Following treatment, the culture medium was discarded and cells were rinsed three times with phosphate-buffered saline (PBS). Cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) was added and the cells were scraped off the dishes and maintained on ice. Cell lysates were placed on ice for 30 min prior to centrifugation at 12,000 x g for 10 min at 4°C. Protein concentration was determined using the bicinchoninic acid assay (BCA) method. The cell lysates were transferred to a 96-well plate, and 200  $\mu$ l BCA working solution was added to each well. After a 30 min incubation at 37°C, the absorbance at 562 nm was determined using a microplate reader. A standard curve was generated and used to determine the concentrations of the samples. The samples were then separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gels), and transferred to a nitrocellulose membrane. The membranes were blocked with 1X PBS-Tween 20 (PBST) solution with 5% bovine serum albumin (BSA; Beyotime Institute of Biotechnology) for 1 h at room temperature. The membranes were then incubated with primary antibodies against MMP-9 (cat. no. ab119906), type IV collagen (cat. no. ab19808), type V collagen (cat. no. ab114072) and  $\beta$ -actin (cat. no. ab8227; all at a dilution of 1:1,000, and obtained from Abcam, Cambridge, MA, USA) overnight at 4°C. The membranes were rinsed three times with PBST (5 min per rinse) and then incubated with a horseradish peroxidase-conjugated secondary antibodies (cat. nos. ab6789; ab 6721) at 1:5,000 dilution for 2 h at room temperature. Finally, the membranes were rinsed with PBST three times (5 min per rinse) and visualized using Pierce ECL Western Blotting substrate (cat. no. 32106; Thermo Fisher Scientific, Inc.).

**Immunofluorescence staining.** Cover slides were placed in 12-well plates, coated with 0.1% poly-lysine (Sigma-Aldrich, Merck Millipore) overnight and rinsed three times with sterile distilled water. Cells were seeded into 12-well plates at a density of  $1.0 \times 10^5$  cells/well, cultured until they reached 70% confluence, and then treated with hemolysate and SB-3CT, as described above. Following the treatment, the medium was removed and the cells were rinsed three times with PBS and fixed in 4% paraformaldehyde for 20 min. The cells were rinsed with PBS and blocked with PBS with 5% BSA for 30 min at room temperature. Following blocking, the cells were incubated with a 1:1,000 dilution of primary antibody against  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; a marker for cerebrovascular smooth muscle cells; cat. no. ab7817; Abcam), MMP-9, type IV and V collagen (Abcam) overnight at 4°C. The cells were then washed three times with PBS and incubated with fluorescent, labeled secondary antibodies (cat. nos. ab150077; ab150115; Abcam) at room temperature for 30 min. Following three additional washes using PBS, the cell nuclei were stained using 4',6-diamidino-2-phenylindole. The slides were then sealed and observed under a BX50/BX-FLA/DP70 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

**Collagen gel contraction assay.** Cell contraction assay kits were purchased from Cell Biolabs, Inc. (San Diego, CA, USA) and used according to the manufacturer's protocol. Following

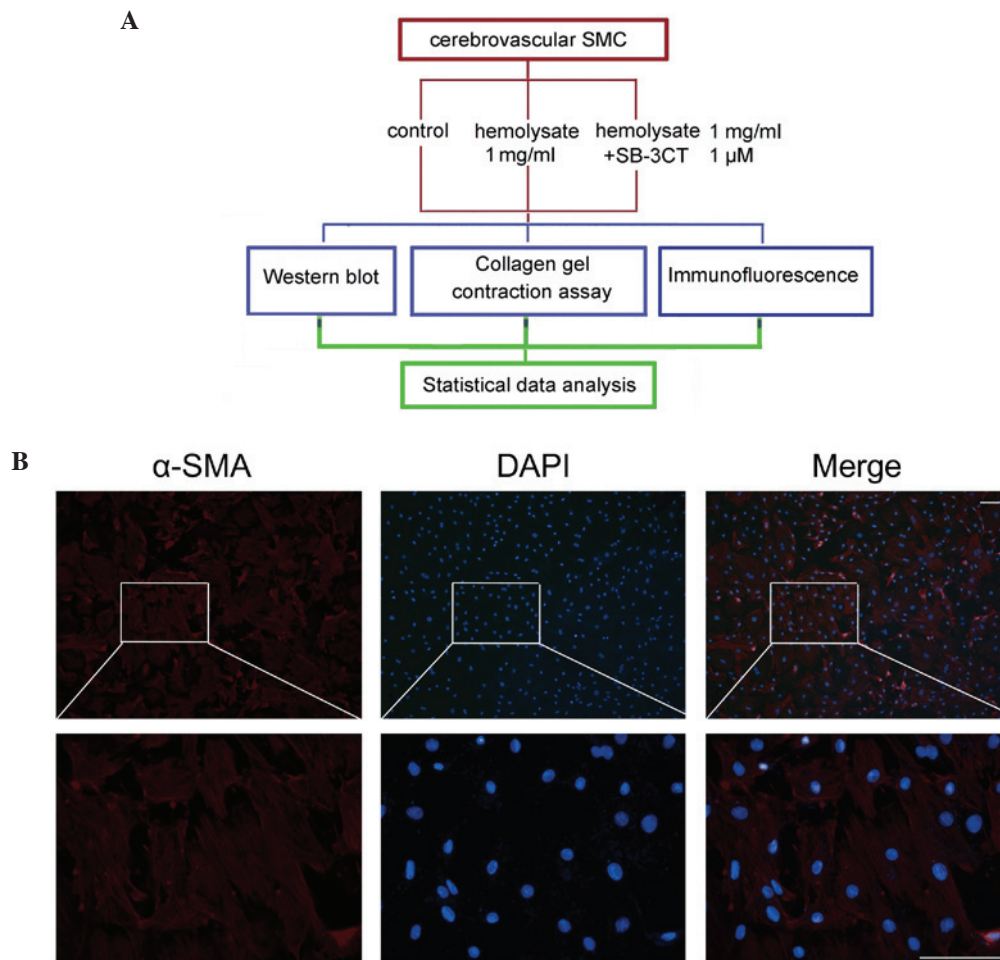


Figure 1. Experimental design and identification of cerebrovascular SMC. (A) Experiments were designed to detect changes in expression levels of MMP-9, collagen IV and collagen V in the cerebrovascular SMC following hemolysate treatment. (B) Identification of cerebrovascular SMC. Red fluorescence indicates staining for  $\alpha$ -SMA, which is a specific marker for cerebrovascular SMC. Blue fluorescence indicates nuclei stained with DAPI. Magnification of the upper panels, x100; that of the lower panels, x400. Scale bar, 50  $\mu$ m. SMC, smooth muscle cells; SB-3CT, 2-[(4-phenoxyphenylsulfonyl)methyl]thiirane;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; MMP-9, matrix metalloproteinase 9.

treatment (Fig. 1), cells were trypsinized and suspended, rinsed three times with PBS, centrifuged at 1,500  $\times$  g for 3 min and resuspended in phenol red-free DMEM (Thermo Fisher Scientific, Inc.) at a final concentration of  $5.0 \times 10^6$  cells/ml. Rat tail collagen solution (Gibco; Thermo Fisher Scientific, Inc.) was diluted to a final concentration of 3 mg/ml with 0.3 ml phenol red-free DMEM (pH 7.3-7.4). Collagen solution, cell suspension and FBS were then combined at a ratio of 8:1:1 to yield a final concentration of  $5.0 \times 10^5$  cells/ml. Aliquots (1 ml/well) of the collagen-cell mixture were added to a 24-well plate and the plates were incubated for 30 min at 37°C with 5% CO<sub>2</sub> to promote polymerization. Following the solidification of the gel, the edges of the gel attached to the wells were scored using pipette tips and an appropriate quantity of phenol red-free DMEM was added. Gels were incubated for an additional 24 h prior to image capture. Cell contractility was calculated using the following formula: Contraction index = (well area - gel area) / well area  $\times$  100%.

**Statistical analysis.** The western blotting and immunofluorescent staining were analyzed using ImageJ version 1.46 software (National Institutes of Health, Bethesda, MD, USA). The mean grayscale value of the control group was normalized to 1, and the ratios of the grayscale values of the experimental groups

to the control group were calculated and analyzed. Statistical analysis was performed using GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean  $\pm$  standard error. One-way analysis of variance and Fisher's least significant difference test was used for pairwise comparison of groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Isolation and culture of cerebrovascular smooth muscle cells.** Immunofluorescence staining was performed to determine the phenotype of the adherent cells. As presented in Fig. 1B, the rat cerebrovascular smooth muscle cells had a polygonal or long, spindle-like morphology and expanded into a monolayer when observed under a microscope. It was demonstrated that the majority of isolated cells stained positively for  $\alpha$ -SMA, a specific marker of cerebrovascular smooth muscle cells. This indicated that the cerebrovascular smooth muscle cells had been successfully isolated (Fig. 1B).

**Hemolysate enhances the contractility of cerebrovascular smooth muscle cells.** As presented in Fig. 2, the contractility of



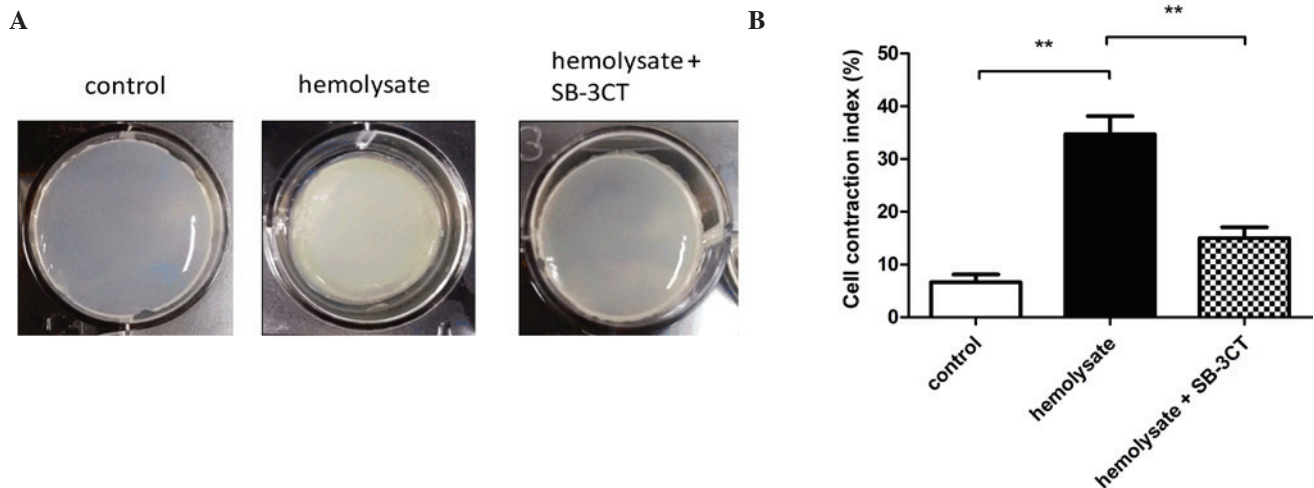


Figure 2. Hemolysate enhances the contractility of cerebrovascular smooth muscle cells. (A) Images of gel contraction following treatment with 1 mg/ml hemolysate and (B) quantification of the gel contraction. Data were derived from three independent experiments. \*\* $P < 0.01$  vs. control group. SB-3CT, 2-[(4-phenoxyphenyl)sulfonyl]methyl]thiirane.

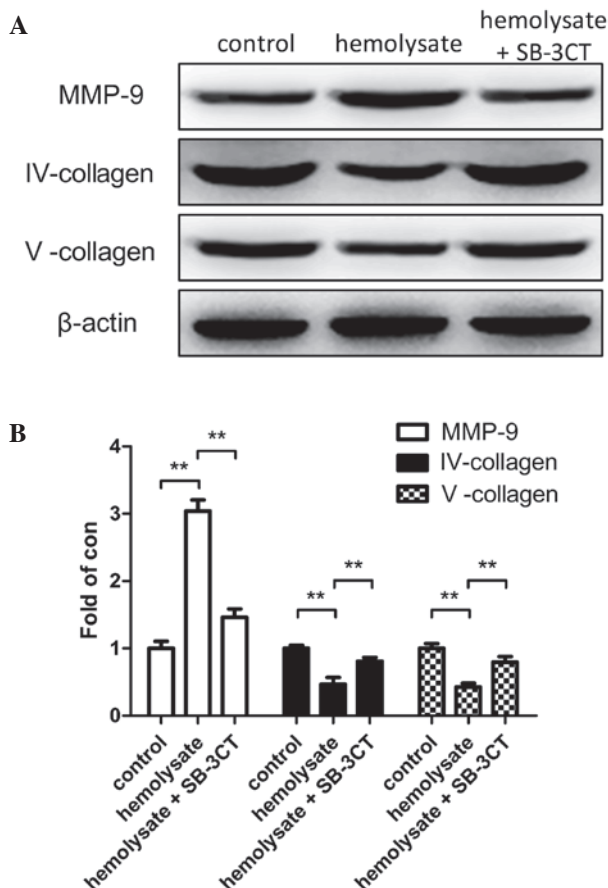


Figure 3. Western blot analysis demonstrated that hemolysate upregulates MMP-9 expression in cerebrovascular smooth muscle cells. (A) Western blot analysis of MMP-9 and collagen IV and V. (B) Quantification of the western blotting. Data were derived from three independent experiments. \*\* $P < 0.01$  vs. control group or the hemolysate + SB-3CT group. MMP-9, matrix metalloproteinase 9; SB-3CT, 2-[(4-phenoxyphenyl)sulfonyl]methyl]thiirane.

cerebrovascular smooth muscle cells was determined using a collagen gel contraction assay. Significant gel contraction was observed in the hemolysate treatment group when compared

with the control group ( $P < 0.01$ ; Fig. 2B). However, pretreatment with SB-3CT significantly inhibited the hemolysate-induced gel contraction when compared with the hemolysate treatment group ( $P < 0.01$ ; Fig. 2B).

*Hemolysate increases MMP-9 protein expression levels in cerebrovascular smooth muscle cells.* Western blot analysis was used to demonstrate that hemolysate stimulation significantly increased MMP-9 protein expression levels ( $P < 0.01$ ; Fig. 3) and reduced collagen IV and V protein expression levels ( $P < 0.01$ ; Fig. 3B) when the hemolysate treatment group was compared with the control. However, pretreatment of cerebrovascular smooth muscle cells with SB-3CT significantly reduced the expression levels of MMP-9 when compared with the hemolysate treatment group ( $P < 0.01$ ; Fig. 3B). Conversely, collagen IV and V protein expression levels were significantly greater in the SB-3CT pretreatment group compared with the hemolysate group ( $P < 0.01$ ; Fig. 3B).

Immunofluorescence staining also demonstrated that hemolysate treatment significantly increased the protein expression levels of MMP-9 ( $P < 0.01$ ; Fig. 4) and reduced the protein expression levels of collagens IV and V ( $P < 0.01$ ; Fig. 4) in cerebrovascular smooth muscle cells compared with the control group. However, pretreatment with SB-3CT significantly reduced the MMP-9 expression levels ( $P < 0.01$ ; Fig. 4D). Collagen IV and V protein expression levels were significantly increased in the SB-3CT pretreatment group compared with the hemolysate group ( $P < 0.01$ ; Fig. 4).

## Discussion

Intracranial aneurysm is a common cerebrovascular disease. Aneurysm re-rupture and CVS are two major complications that may occur following a ruptured aneurysm. CVS occurs at a rate of 30-70%, with ~30% of these patients presenting symptoms of cerebral ischemia or severe cerebral infarctions (1). As the pathogenesis of CVS remains to be fully elucidated, the treatment options are currently limited. Therefore, prevention of CVS is crucial for the reduction of

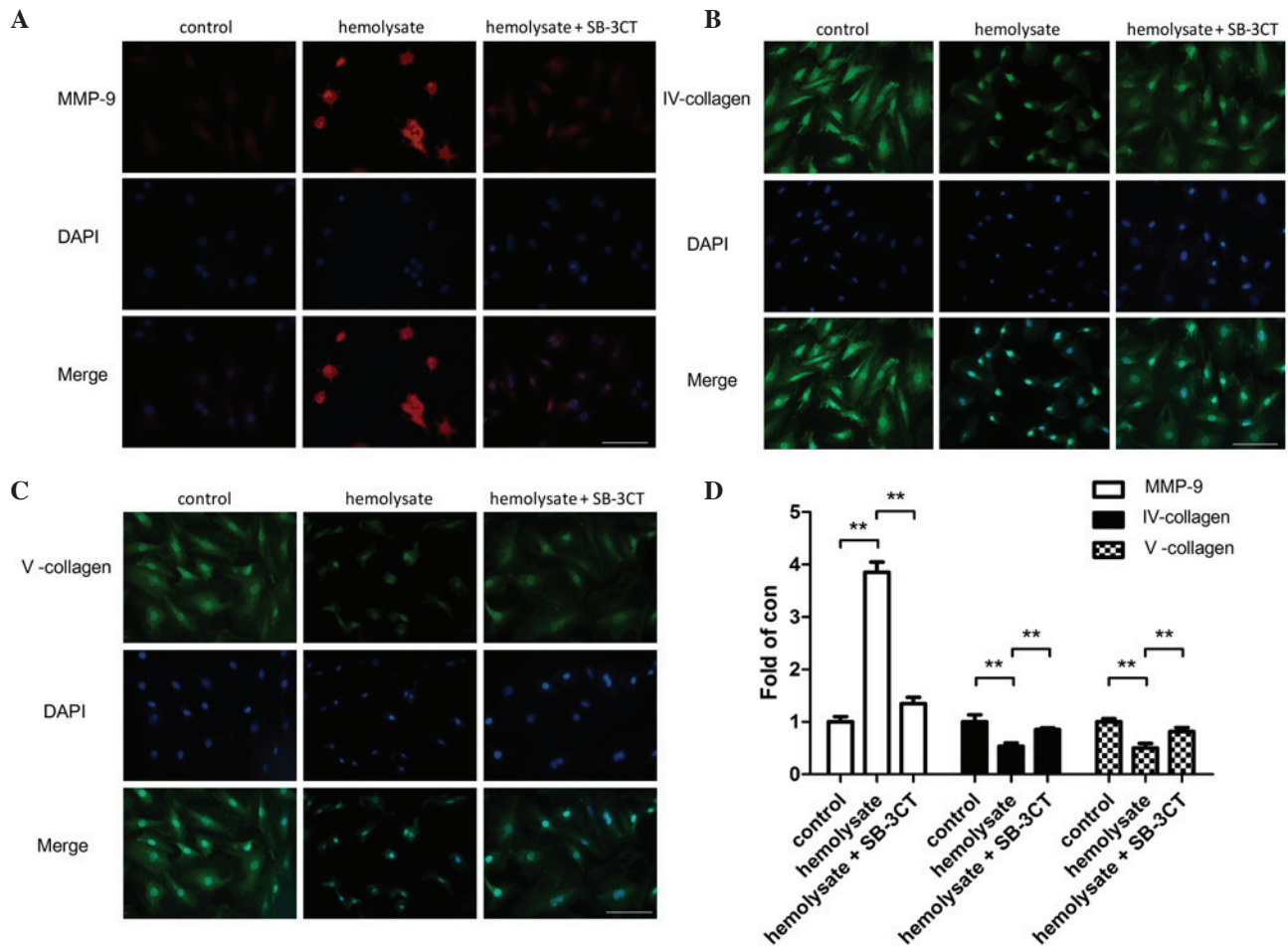


Figure 4. Immunofluorescent staining demonstrated that hemolysate upregulates the expression levels of MMP-9 in cerebrovascular smooth muscle cells. Immunofluorescent staining for (A) MMP-9, (B) collagen IV and (C) collagen V following treatment with 1 mg/ml hemolysate with and without pretreatment using 1  $\mu$ M SB-3CT. Magnification, x400. Scale bar, 50  $\mu$ m. (D) Quantification of immunofluorescent staining. Data were derived from three independent experiments. \*\* $P$ <0.01 vs. control group or the hemolysate + SB-3CT group. MMP-9, matrix metalloproteinase 9; SB-3CT, 2-[(4-phenoxyphenylsulfonyl)methyl]thiirane; DAPI, 4',6-diamidino-2-phenylindole.

morbidity and mortality rates following intracranial aneurysm surgery.

Previous studies have determined that CVS may be due to multiple factors that enhance cerebral vasoconstriction (2,4) and diminish cerebral vasodilation (3,5,11). Humphrey *et al* (11) proposed a theoretical biomechanical framework of SAH vasospasm (11), which noted that, following SAH, extracellular clot stimulation of blood vessel walls led to increased levels of NO scavengers, such as reactive oxygen species and oxyhemoglobin, including higher levels of serotonin, thromboxane A<sub>2</sub>, angiotensin-I and thrombin. This, in turn, may lead to increased vascular thickness and hardness, stenosis and vasoconstriction. The vessel may return to normal when the clot dissolves. Different treatment strategies have been evaluated based on these mechanisms. Previous studies have determined the effects of several antagonists on the contraction of cerebral smooth muscle, including calcium channel blockers such as papaverine (12) and nicardipine (13). Fenpropathrin povidone (14) and endothelin antagonists (15) were also revealed to have a therapeutic effect. However, the overall effectiveness of these treatments was unsatisfactory as of the exact pathogenesis of remains to be elucidated.

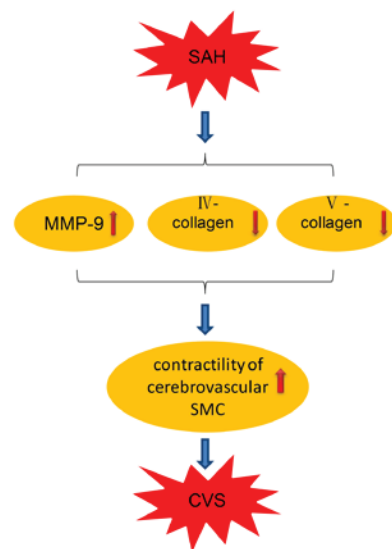


Figure 5. Hypothesized model for the importance of MMP-9 in cerebrovascular SMC following SAH. MMP-9 expression levels were increased in cerebrovascular SMC, whereas expression levels of collagen IV and V decreased significantly following SAH. These changes led to enhanced contractility in cerebrovascular SMC and led to CVS. SAH, subarachnoid hemorrhage; MMP-9, matrix metalloproteinase 9; SMC, smooth muscle cells; CVS, cerebral vasoconstriction.

Previous studies have determined that MMP-9 may be associated with cerebrovascular disease. MMP-9 may degrade the extracellular matrix (16-21). Additionally, it may be involved in various pathological processes, including the degradation of the connective tissue, inflammation, ischemia and hypoxia. Therefore, MMP-9 is important for pathological processes associated with cerebral hemorrhage. Compared with previous studies on cerebral hemorrhage, the effects of MMP-9 on aneurysmal SAH remain to be fully elucidated. However, the current study identified MMP-9 as a potential biomarker for CVS following an aneurysm (16). Previous studies have determined that, following aneurysmal SAH, the expression levels of MMP-9 are increased in brain tissue, cerebrospinal fluid and peripheral blood (17-19). Therefore, MMP-9 is important in the pathological processes of aneurysmal SAH (17,20,21).

Our previous study on a rat model revealed that CVS may occur following SAH (9). Additionally, protein expression levels of MMP-9 in the basilar artery walls increased following SAH with a parallel time course to development of CVS. This suggested that MMP-9 may be involved in the pathological processes of CVS (9). CVS primarily occurs due to enhanced contractility of cerebrovascular smooth muscle cells. The aim of the present study was to determine whether MMP-9 may be involved in this process (Fig. 5). Cerebrovascular smooth muscle cells were treated with hemolysate as an *in vitro* model of SAH. Collagen gel contraction experiments revealed that hemolysate treatment induced a significant contractile response in cerebrovascular smooth muscle cells. In addition, pretreatment with SB-3CT (a selective inhibitor of MMP-9) reduced these contractile responses. Immunofluorescence staining and western blotting confirmed that hemolysate stimulation increased MMP-9 protein expression levels in cerebrovascular smooth muscle cells. In contrast, protein expression levels of collagen IV and V were significantly decreased. This suggests that MMP-9 contributed to protease activity, catalyzed the degradation of collagen IV and V and was important for the contractile response of cerebrovascular smooth muscle cells. Additionally, pretreatment with SB-3CT inhibited the protease activity of MMP-9 and the contractile responses of the cerebrovascular smooth muscle cells.

In conclusion, the present study used an *in vitro* model of SAH to investigate changes in MMP-9 expression levels in cerebrovascular smooth muscle cells, and its involvement in their contractile response. Therefore, the current study provided novel insights into the pathogenesis of CVS following SAH. Further research is necessary to elucidate the specific molecular mechanisms that mediate the effects of MMP-9 on the contraction of cerebrovascular smooth muscle cells, and whether MMP-9 is involved in cerebral inflammation following SAH.

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