A Novel Animal Model for Subarachnoid Hemorrhage in Rabbits - Pathology due to Hemorrhage

by ***Lin Wang, Meng-liang Zhou, Hui-lin Chen, Wei Wu, Hong-xia Yin & Ji-xin Shi****
Neurosurgery Department, Jinling Hospital, Medical School of Nanjing University, Jiangsu Province, China

Abstract
The Pathophysiological course of subarachnoid hemorrhage (SAH) is not completely understood, such as how and why blood or its lysate can influence the brain. Although many SAH animal models have previously been reported, none are suitable for research in this field. The aim of the present study was to establish such an animal model. Twenty New Zealand rabbits were divided into two groups, a control group and a SAH group (n=10). Blood was lysed using an *in vitro* freeze-thaw method. After anesthesia, 0.9 ml of cerebrospinal fluid was drawn from both groups. An identical volume of heparinized hemolysate was then slowly injected into the *cisterna magna* of the SAH group animals. Control group animals while the control group received heparinized isotonic sodium chloride solution. Forty-eight hours post-injection, samples were excised after perfusion fixation for further determinations. The degree of cerebral vasospasm was evaluated by measuring the cross-sectional area of the basilar arterial lumen, and brain damage was examined by TUNEL staining. Weight, blood pressure and saturation of blood oxygen showed no statistical differences between groups. The cross-sectional area of the arterial lumen in the SAH group was roundly 0.402 mm^2, which was statistically the same as that for the control group (0.435 mm^2). The apoptosis index for the SAH group was significantly higher than that for the control group. Taken together, our results show that this model is effective in reproducing the influence of blood on the brain after SAH and, at the same time, strictly excludes influences on the brain by other factors. Thus, this model can effectively be applied to investigate blood-related neuropathophysiology following SAH.

Introduction
Subarachnoid hemorrhage (SAH) is a relatively common condition with a poor outcome, where 20%-35% of SAH patients die within a few days (*Bonita et al., 1985*). Unfortunately, an effective therapy that specifically targets the pathophysiological mechanism of SAH does not currently exist. A major obstacle in developing SAH therapy is the paucity of knowledge involving underlying SAH mechanisms. There are many clinical and experimental studies on SAH that have focused mainly on SAH-induced cerebral vasospasm. However, whether or not cerebral vasospasm is the only factor that affects the outcome remains open. Treatments that focus purely on cerebral vasospasm have not yielded satisfying positive effects. Also, cerebral vasospasm fails to account for all related clinical manifestations. For example, some SAH patients have slight, if any, symptoms but exhibit obvious cerebral vasospasm; patients can also exhibit severe symptoms with no signs of cerebral vasospasm (*Liu, 2000*). Thus, it is likely that some other pathophysiological mechanism underlies SAH.

It is reasonable to presume that blood or its lysate may directly damage the brain after SAH. Indeed, blood or its lysate has been shown to damage other normal tissues. Sub-retinal hemorrhage induces

**Correspondence:* Ji-Xin Shi
Department of Neurosurgery, Jinling Hospital
305 East Zhongshan Road, Nanjing 210002, P.R.China
Tel: +86-25-85962075
Fax: +86-25-84806839
Email: wanglin1.student@sina.com
wangling_77@hotmail.com

optic nerve atrophy (*Ohno-Matsui et al., 1996*); hemosiderin damages the lungs of patients with thalassanemia (*Yao et al., 2003*); heme, released from red blood cells, is cytotoxic (*Abraham et al., 1995; Hunt et al., 1996; Jeney et al., 2002*); and bilirubin causes central nervous dysfunction in hemolysis patients (*Oygur et al., 1996; Hansen, 2002; Shapiro, 2003*). Reports have shown that blood or its lysate causes pathological changes or inflammation of the basilar artery after SAH (*Wakai et al., 1982; Seifert et al., 1989; Peterson et al., 1990; Dumont et al., 2003*).

Blood or its lysate may also directly damage the brain after SAH since neural cells are vulnerable to harmful factors and their normal function and survival depends on strict homeostasis, including cerebrospinal fluid (CSF) stabilization. Besides protecting the brain from trauma, CSF provides essential nutritive substances to the central nervous system (*Waxman, 1999*). After SAH, and particularly after hemolysis, the properties of CSF change acutely, including pH, osmolality, and ion concentration. Potentially harmful substances then emerge in the CSF, such as complements, iron, hemoglobin etc. Therefore, to better understand and manage SAH, further studies are necessary to investigate the subsequent neural pathophysiological changes caused by blood or its lysate after SAH.

Many SAH animal models with cerebral vasospasm have been reported (*Spallone and Pastore, 1989; Fadel et al., 1995; Megyesi et al., 2000*) using various animals and methods. It is clear that such models cannot be applied to investigate blood-related neuropathophysiology following SAH since vasospasm-related ischemia would confuse the analysis. The purpose of the present study was to create an animal model applicable for the study of the influence of blood on the brain following SAH.

Materials and Methods

Animals

Conventional female New Zealand rabbits, age 5 months and weight 2.4-2.6 kg, were supplied from the Experimental Animal Centre of Jiangsu, Nanjing (China). They were transported by van and acclimatized for 1 week in the Central Animal Laboratory of Jinling Hospital. The rabbits were housed during the experiment in individual galvanized steel wire cages (50 x 50 x 80cm) without bedding. The room had ten air changes per h and temperature and humidity were regulated (25 ± 2°C; 55 ± 10%). Fluorescent lighting was *c*.300 Lux at 2 m above floor from 6.30 to 18.30. Diet provided was untreated 3 x 15 mm pellets (Jiangning Feeds Ltd, Nanjing, China) given 50 g/kg twice per day and tap water with automatic watering system. Experiments were performed from April to June, 2004, using twenty rabbits that were randomly divided into two groups; a control group and a SAH group (n=10). All experimental protocols met the requirements of the Laboratory Animal Care and Use Guidelines published by the Medical School of Nanjing University (available in English).

Hemolysate preparation

Blood cells were lysed with the freeze-thaw method in order to obtain hemolysate (*Matz et al., 2000*). Arterial blood was obtained from the ear central artery with a sterile syringe and needle. The blood was placed into a sterile container and stored at -20°C for 20 min. Hemolysate was immediately thawed in a 39°C water bath for further use. All operations were in strict accordance with asepsic principles.

Experimental subarachnoid hemorrhage

Each animal was anaesthetized with an intramuscular injection of ketamine (25 mg/kg) and droperidol (1.0 mg/kg). The local hair of the neck was sheared and the skin was sterilized with 75% ethanol. The SAH group was subjected to a percutaneous puncture to the *cisterna magna*. After a volume of 0.9 ml cerebrospinal fluid was drawn, an identical volume of 39°C-heated autologous heparinized hemolysate was slowly injected into the *cisterna magna* for 20 seconds. The control group were treated similarly, but received heparinized isotonic sodium chloride

instead of hemolysate. The animals were then placed in a head-down position of 30 degrees for 30 min. After recovery from anesthesia, they were returned to the vivarium.

Perfusion-fixation

Forty-eight h after SAH, animals were again anesthetized with an intramuscular injection of ketamine (40 mg/kg) and droperidol (2.5 mg/kg). The animals were intubated with a 3.5 mm diameter endotracheal tube and mechanically ventilated using a rodent ventilator (SGC, China). A 22-G butterfly needle (BD, China) was placed into the central ear artery and the mean arterial pressure (MAP) and heart rate (HR) were constantly measured with a monitor (Spacelabs Medical, USA). Blood samples were obtained at 0, 15, 30, 45 min to determine blood gas levels. Next, perfusion-fixation was performed as described in earlier studies (*Grasso, 2001; Satoh et al., 2001; Tsurutani et al., 2003*). The thorax was opened, a cannula was placed in the left ventricle, the descending thoracic aorta clamped, and the right atrium opened. Perfusion began with 300 ml of 39°C HBSS, pH 7.4, followed by 400 ml of 10% buffered formaldehyde under a perfusion pressure of 120 cm H2O. The brain was then removed and immersed in the same fixative.

TUNEL staining

Formalin-fixed tissues were embedded in paraffin and sectioned at 4 μm thickness with a microtome. Sections were examined for apoptotic cells using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. The TUNEL: *In Situ* Cell Death Detection Kit POD (ISCDD, Boehringer Mannheim, Germany) was used according to manufacturer's instructions. Briefly, sections were de-paraffinized, re-hydrated, and washed with distilled water. Tissues were digested with 20g/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol in PBS at 37°C for 30 min. Sections were next incubated with terminal deoxynucleotidyl transferase at 37°C for 60 min to add dioxigenin-conjugated dUTP to the 3'-OH ends of fragmented DNA. Anti-dioxigenin antibody peroxidase was applied to the sections for labeled nucleotide detection. The sections were stained with DAB and slightly counterstained with hematoxylin. The positive cells were identified, counted and analyzed under light microscopy by another investigator without knowing the group setting. The extent of neurological damage was evaluated by the apoptotic index, defined as the average number of positive neuronal cells per 100 neuronal cells. At least 1000 neuronal cells were counted.

Measurement of blood vessel cross-sectional area

According to the previous reports (*Kwan et al., 2001; Lin et al., 2001; Ogane et al., 2002; Sonmez et al., 2002; Tsurutani et al., 2003*), the degree of cerebral vasospasm can been evaluated by measuring cross-sectional areas of the basilar artery lumen. The formalin-fixed basilar artery was embedded in paraffin, sectioned at 4 μm thickness with a microtome and stained with hematoxylin and eosin. Micrographs of the basilar artery were taken and entered into a computer database. The cross-sectional area of the blood vessels was determined by an investigator who did not know the group setting, using the High Definition Medical Image Analysis Program (HMIAP-2000, developed by Tongji Medical University, China). The area was calculated by measuring the perimeter of the actual vessel lumen and then calculating the area of an equivalent circle (area=π r2, where r= radius) based on the calculated equivalent r value from the perimeter measurement (r= perimeter/2π), thus correcting for vessel deformation and off-transverse sections. For each vessel, at each mid-point of the proximal, the middle and the distal third, three sequential sections were taken, measured and averaged. Results here are expressed as the mean.

Statistical analysis

Software SPSS 11.0 was used for statistical analy-

sis. According to the type of data property, the independent-sample *t*-test or non-parametric test was used, respectively. The level of significance was denoted as $p \leq 0.05$.

Results

General observation

The experimental rabbits presented in the study were less active and anorexic after SAH when compared to control rabbits. The SAH animals did not exhibit any clots over the basal surface of the brainstem but obvious bloodstaining at the base of the temporal lobe was observed (Fig.1).

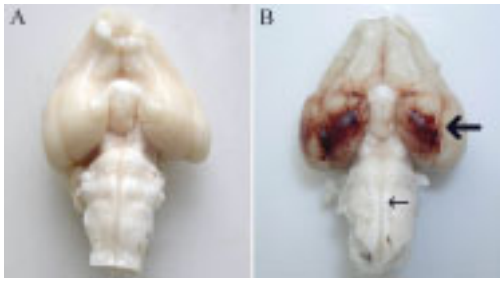

Figure 1. Ventral view of typical brains from control (A) and subarachnoid hemorrhage (SAH) (B) groups. The image in (B) shows a bloodstain at the base of the temporal lobe and the absence of clots over the brainstem. The image in (A) does not show indications of bloodstains or clots.

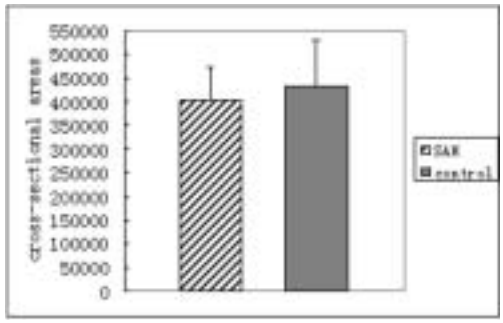

Figure 2. Comparison of the mean cross-sectional areas (μm^2) obtained from the basilar artery of the subarachnoid hemorrhage (SAH) and control groups. No statistical differences between the mean areas for the groups were observed (p=0.455, independent-samples *t*-test).

Physiological parameters

In the course of perfusion fixation, continuous monitoring of MAP (mean arterial pressure) showed that some operations could induce a rise of MAP in the range of 30 mmHg and then return to baseline value in a short time. The mean ± SD of MAP and blood gas results for the different groups are shown in Table 1. No significant differences between the two groups (using an independent-samples t-test) were observed. These results indicate that it is unlikely that there is any neural damage caused by hypoxia, ischemia, acidosis or alkalosis.

Table 1. Physiological parameters of the two groups during the operation. Abbreviations: MAP= mean arterial pressure, pCO_2= partial pressure of carbon dioxide; pO_2= partial pressure of oxygen, sO_2= saturation of blood oxygen. All values are expressed as mean ± SD. P values indicate there are no statistical differences between groups.

Group	MAP (mmHg)	pH (mmHg)	pCO_2 (mmHg)	pO_2	sO_2
Control	76.10±8.16	7.457±0.076	36.1±4.6	81.7±7.99	0.965±0.011
SAH	79.40±9.17	7.443±0.059	33.7±3.7	89.0±15.10	0.968±0.017
P	0.406	0.651	0.214	0.193	0.641

Table 2. Mean cross-sectional areas of basilar arteries of the two groups.

Group	Cross-sectional areas (μm2)									
Control	339700	439800	581800	322800	66200*	374900	553800	108600*	483400	382400
SAH	438500	474300	430600	346700	380900	54600*	504900	286400	352100	87900*
*these data were beyond the range of mean ± 3SD, therefore they were eliminated;										

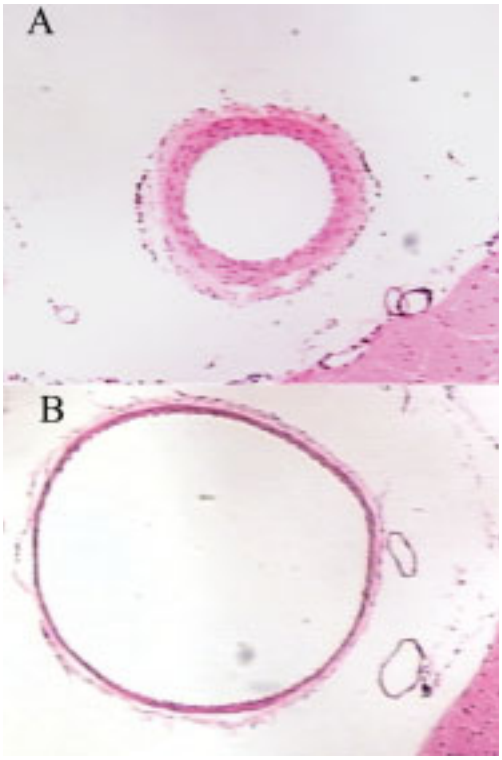

Figure 3. Morphological observation of basilar artery (HE staining). A is from a spastic rabbit basilar artery, whose internal elastic lamina (IEL) is corrugated, the wall thick and the lumen small. B is a basilar artery from a rabbit examined in the present study, indicating an IEL that is smooth, a wall thin and a lumen is large. The basilar arteries of the subarachnoid hemorrhage (SAH) and control groups are morphologically similar (as in B) and do not have any spastic artery characteristics. This supports the fact that at the time of perfusion there was no vasospasm.

Evaluation of cerebral vasospasm

Cross-sectional areas for all animals are shown in Table 2. Four sets of data fell beyond the range of mean ± 3 SDs, so they were thought not to be from the group and were counted in the statistical analysis (this is called "the three s principle" in statistics). The mean cross-sectional area of the SAH group was 401800±73019μm2, indicating no significant difference to that of the control group (434825±97159μm2) (Fig. 2). Furthermore, the arteries observed in this study did not exhibit any morphological characteristics of a spastic artery (Fig.3). Thus, blood supply did not show an evident statistical difference between the two groups and the animals did not have cerebral vasospasm.

Apoptosis

In the SAH group, TUNEL staining showed obvious apoptotic cells. Furthermore, these cells were mainly located at the base of the temporal lobe. In the control group, there were few, if any, apoptotic cells irregularly distributed within the brain tissue (Fig. 4). The apoptosis index of the SAH group ranged from 3/100 to 42/100, and the control group 0/100 to 4/100 (Table 3). The apoptosis index of the SAH group was significantly larger than that of the control group (p=0.001, Mann-Whitney U test), an indication that the brain was obviously damaged after SAH.

Discussion

A model for investigating blood-related neuropathophysiology after SAH should fulfill the following criteria: 1) the model should be able to reproduce the influence of the blood on the brain after SAH, and 2) the model should exclude influ-

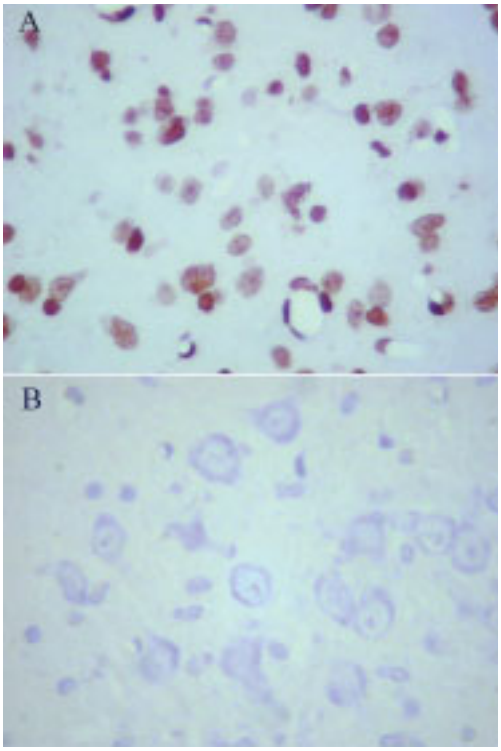

Figure 4. Histopathological micrographs of rabbit brain cortex (TUNEL staining, x400). A is a section from the subarachnoid hemorrhage (SAH) group; the cell (black arrow) is dystrophic and brown-stained, indicative of an apoptotic cell. B is one from the control group; the cell (blank arrow) has normal structure and is lightly blue-stained, indicative of a normal cell.

ences by other factors on the brain, including cerebral vasospasm, high intracranial pressure, ischemia, anoxemia, *etc*. The main problem in creating a working model is how to avoid the occurrence of cerebral vasospasm while at the same time, keeping enough of the blood's influence on the brain.

Rabbits were chosen for this study since the rabbit SAH model has a steady time course of cerebral vasospasm, usually beginning at the third day (*Megyesi et al., 2000*). We chose 48 h as an observation time in order to reduce the probability of cerebral vasospasm and simultaneously ensure enough time for the blood to cause neural pathophysiological changes. In previous studies, the "two-hemorrhage" model was frequently used because it enhanced stimulation to blood vessels and increased the probability of vasospasm (*Spallone and Pastore, 1989; Fadel et al., 1995; Megyesi et al., 2000*). To reduce the probability of cerebral vasospasm, we adopted a "single-hemorrhage" model. Typically, others injected 1.2-3.0 ml blood into the *cisterna magna* (*Spallone & Pastore, 1989; Fadel et al., 1995; Megyesi et al., 2000*). In order to prevent cerebral vasospasm, ere reduced the injection volume. However, extreme care was taken so that the injection volume was not too small to cause neural pathophysiological changes. Therefore, 0.9 ml was chosen as the injection volume; in preliminary experiments, that quantity reduced stimulation on vessels but was enough to cause obvious brain changes. Blood was heparinized to prevent blood clot formation since this mechanical stimulation on blood vessels can contribute to cerebral vasospasm. Macroscopic observation showed no clots near the basilar artery but an obvious bloodstain at the base of the tempo-

Table 3. Brain apoptosis index of control and SAH groups

Group	Brain apoptosis index*							
control	0	0	4	0	0	0	0	1
SAH	42	22	16	28	3	24	29	7

*apoptotic index is defined as the average number of positive neuronal cells per 100 neuronal cells. At least 1000 neuronal cells were counted.

ral lobe was seen (Fig. 1). It is likely that with the 30 min head-down position, the lysate concentrated at the middle cranial fossa. Furthermore, the upflow of CSF from the *cisterna magna* to the top side of the brain may contribute to the lysate's concentration at the middle cranial fossa. The stimulation to the blood vessel is slight; however, the stimulation to the neural tissue is relatively intense. There, the model is very suitable for studying neurological changes directly caused by blood or its lysate.

Blood lysate was injected instead of blood itself in order to mimic an actual SAH condition. After an aneurysm ruptures, blood cells begin to lyse in the CSF from the third day (*Peterson et al., 1990*). Therefore, the presence of iron, potassium, hemoglobin and oxidation enzymes in the subarachnoid space was not a factor within our observation time of 48 h. Thus, the blood lysate was injected to better reproduce an actual blood influence on the brain. The injection volume was identical to the volume drawn in order to avoid high intracranial pressure that could reduce perfusion pressure and induce neural ischemic changes. To investigate blood-related neural pathophysiological changes, high intracranial pressure should be avoided, though this is more analogous to the clinical condition. Other factors that may damage the brain were also monitored, including blood pressure, saturation of blood oxygen, and blood pH value.

Comparison of the basilar artery area between the two groups showed no statistical difference between the groups. Furthermore, the morphological study verified that there were no obvious spastic vessels: the internal elastic lamina (IEL) of the vessels in the SAH animals were smooth, the walls thin and the lumen large. This was morphologically different from a spastic vessel whose IEL was corrugated, the wall thick and the lumen small (*Fadel et al., 1995; Kiris et al., 1999; Barbosa et al., 2001; Satoh et al., 2002; Sonmez et al., 2002*). Thus, there is no evidence of cerebral vasospasm in this study. There are no reports that focus on whether ketamine or droperidol can attenuate cerebral vasospasm after SAH. Both of these two anesthetics have often been previously applied in SAH experiments (*Lin et al., 2001; Gabikian et al., 2002; Watanabe et al., 2005*). If these anesthetics could attenuate cerebral vasospasm, evidence would have been documented that supported this; thus far, there is no evidence to the contrary. Thus, there is little possibility that the anesthetic combination used in this study would resolve any cerebral vasospasm that may have been present. Our results, using our novel SAH animal model, indicated that cerebral vasospasm-related brain damage was not present. Blood pressure and blood gas analysis indicated no differences between the two groups.

This model is not suitable unless there is evidence that shows some neural pathophysiological changes are directly caused by blood or its lysate. TUNEL staining showed the animals from the SAH group had obvious brain damage. Because there was no influence from hypoxemia, low blood pressure, high intracranial pressure or vasospasm-related ischemia, the brain damage was most likely directly induced by the blood or its lysate. After SAH, inflammation will present in response to subarachnoid blood (*Peterson et al., 1990*). It could induce release of inflammatory cytokines, including interleukin (IL)-1beta, IL-6, and tumour necrosis factor (TNF)-alpha (*Fassbender et al., 2001*). Reports have shown that IL-1 on TNF-alpha can induce cell apoptosis (*Ayala et al., 1997; Williams et al., 1997*). Thus, the cell apoptosis in the cortex may be related to the inflammation following SAH.

In conclusion, our model effectively reproduces the influence of blood on the brain after SAH and it also strictly excludes influences by other factors on the brain, including cerebral vasospasm, high intracranial pressure, low blood pressure, hypoxemia, alkalosis, and acidosis. Thus, our animal model is suitable for investigations into blood-related neuropathophysiology following SAH. It should be noted that our SAH model is not useful for study in other fields since it does not appear to cause cerebral vasospasm, high intracranial pressure, or blood clot formation surrounding a vessel, characteristics often present in the clinical condition. This model also has the possible limitation that the

freeze-thaw process may denature some proteins, although the temperature range from –20°C to 39°C is relatively gentle. Perhaps some toxic matter was eliminated in this process, such as the RBC surface protein. Therefore, should this model be used in experimental investigations, these specific limitations should be considered.

SAH is a relatively common condition that has considerable mortality and morbidity. Because of its poor prognosis and because there is currently no effective management for SAH, new ideas and new models should be introduced and investigated in an attempt to improve the SAH situation. Our novel SAH animal model characterized here is a first step in investigating the blood-related neuropathophysiology after SAH and may serve as a useful tool for further study in this new field.

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