An Experimental Model of Symptomatic Vasospasm Induced by Oxyhemoglobin in Rabbits

Tsuneo Otsuji, MD; Shunro Endo, MD; Yutaka Hirashima, MD; Michiharu Nishijima, MD; Akira Takaku, MD

Background and Purpose: There are many experimental models for studies of cerebral vasospasm. However, no ideal model has been established thus far to comparatively reproduce the ischemic state of the brain that may occur in patients after subarachnoid hemorrhage.

Methods: In the present study, we attempted to induce severe vasospasm in rabbits by using an oxyhemoglobin-rich blood product prepared from hemolyzed arterial blood and evaluate neurological symptoms, cerebral angiogram, cerebral blood flow, and histology.

Results: Clinically significant neurological symptoms were observed in about half of the rabbits. There was no significant correlation between angiographic results of the vasospastic state of the main artery and the severity of neurological symptoms observed. However, the cerebral blood flow was significantly lower than in the control group and significantly correlated with the severity of neurological symptoms. On histological examination, lesions were found in about half of the rabbits. Development of obvious infarction was found more frequently than in other reported models.

Conclusions: These results suggest that this model is appropriate as an experimental model of vasospasm occurring after subarachnoid hemorrhage and is especially useful in that it induces vasospasm intense enough to cause obvious infarction.

Key Words: animal models • oxyhemoglobin • subarachnoid hemorrhage • vasospasm • rabbits

Materials and Methods:

Thirty-six Japanese White rabbits of 2.5 to 3.0 kg body weight were used in this study. Thirty-one animals were treated in advance by bilateral CCA ligation. Among them, 3 were excluded from this study (see below), and 23 underwent induced SAH (SAH group). The remaining 5 rabbits with CCA ligation and 5 rabbits without ligation were defined as CCA ligation group and untreated group, respectively. Experimental SAH was induced using two injections of autologous arterial blood (first) and OxyHb (second) into the cisterna magna at an interval of 2 days. The outline of the protocol is shown in Fig 1. The day of the first blood injection was set as day 0. Rabbits were neurologically followed, and the changes in cerebral angiogram and local cerebral blood flow (CBF) were investigated. For neurological evaluation, the animals were observed on a flat floor. Manual muscle testing was also done. Combining the assessment results, neurological symptoms were categorized into four grades (Table 1). The measurement of CBF was also performed in the 10 rabbits of the CCA ligation and untreated groups.

On completion of these studies, all animals were exsanguinated for histological examination. Table 2 shows the number of evaluated animals. During surgery, angiography, and measurement of CBF, the animals were anesthetized with ketamine hydrochloride 50 mg/kg IM. All protocols were approved by the Animal Ethics Committee of the Toyama Medical and Pharmaceutical University. The details of each experimental procedure are as follows.

Bilateral CCA Ligation

The animals were placed in a supine position. Right and left CCAs were approached in a sterile manner through a 1.5-cm median incision in the cervical region. The CCA was bilaterally ligated with double knots of silk tie, and the skin wound
TABLE 1. Grading of Neurological Deficit in This Study

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No deficit (normal)</td>
</tr>
<tr>
<td>2</td>
<td>Minimum or suspicious deficit</td>
</tr>
<tr>
<td>3</td>
<td>Mild deficit without abnormal movement</td>
</tr>
<tr>
<td>4</td>
<td>Severe deficit with abnormal movement</td>
</tr>
</tbody>
</table>

TABLE 2. Number of Rabbits In This Study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CCA Lig</th>
<th>SAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiography</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>CBF</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Histology</td>
<td>2</td>
<td>23</td>
</tr>
</tbody>
</table>

CBF indicates cerebral blood flow; CCA Lig, common carotid artery ligation; and SAH, subarachnoid hemorrhage.

was closed. The rabbits with both CCAs ligated stayed under observation for the following 2 weeks. Animals were excluded from the study if they died, developed neurological symptoms, or lost their appetite severely during the observation period.

FIG 1. Graphic of experimental protocol of this study. The common carotid arteries had been ligated bilaterally 2 weeks before the first induction of subarachnoid hemorrhage. Two days after the first induction, oxyhemoglobin-rich blood derivative (OxyHb) was given as the second induction. CBF Indicates cerebral blood flow.

Injection of Blood and OxyHb

The animals were placed in a prone position, and a midline incision was made in a sterile manner from the occipital protuberance to 1.5 cm caudally. The interosseus membrane between the first cervical vertebra and the occipital bone was

TABLE 3. Summary of Results of Experimental Subarachnoid Hemorrhage in 23 Rabbits

<table>
<thead>
<tr>
<th>Neurological Grade</th>
<th>Rabbit</th>
<th>Neurology</th>
<th>Angiographic Diameter Reduction, %</th>
<th>CBF Average (right/left)</th>
<th>Histology</th>
<th>Date of Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 2</td>
<td>1</td>
<td>Suspicious deficit</td>
<td>-28.0</td>
<td>39.2 (41.5 /37.9)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Suspicious deficit</td>
<td>...</td>
<td>37.6 (35.5 /36.9)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Suspicious deficit</td>
<td>...</td>
<td>...</td>
<td>Cortex delayed neuronal death</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Suspicious deficit</td>
<td>-11.5</td>
<td>42.3 (43.3 /41.3)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Suspicious deficit</td>
<td>-35.2</td>
<td>36.3 (33.0 /39.6)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Suspicious deficit</td>
<td>-24.4</td>
<td>38.3 (38.5 /38.1)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Suspicious deficit</td>
<td>-24.7</td>
<td>37.7 (38.6 /36.7)</td>
<td>Right hippocampus infarction</td>
<td>Day 4</td>
</tr>
<tr>
<td>Grade 3</td>
<td>8</td>
<td>Left hemiparesis</td>
<td>-12.2</td>
<td>32.2 (31.4 /33.0)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Left hemiparesis</td>
<td>-16.7</td>
<td>32.5 (31.9 /33.0)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Left hemiparesis</td>
<td>-43.7</td>
<td>31.3 (30.9 /31.6)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Lethargy</td>
<td>-32.7</td>
<td>28.3 (29.0 /27.5)</td>
<td>No lesion</td>
<td>Day 4</td>
</tr>
<tr>
<td>Grade 4</td>
<td>12</td>
<td>Tetraparesis</td>
<td>-29.0</td>
<td>22.6 (19.5 /20.0)</td>
<td>Left hippocampus infarction</td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Tetraparesis</td>
<td>-40.1</td>
<td>31.1 (29.9 /32.2)</td>
<td>Pons infarction</td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Tetraparesis</td>
<td>...</td>
<td>29.7 (33.0 /26.4)</td>
<td>No lesion</td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Tetraparesis</td>
<td>-53.4</td>
<td>26.8 (30.5 /23.0)</td>
<td>No lesion</td>
<td>Day 3</td>
</tr>
</tbody>
</table>

Death

<table>
<thead>
<tr>
<th>Date and Time of Death</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 Day 2, just after 2nd Injection</td>
<td>Medulla edema</td>
</tr>
<tr>
<td>17 Day 2, 12 hours after 2nd Injection</td>
<td>Cortex edema</td>
</tr>
<tr>
<td>18 Day 2, 1 hour after 2nd Injection</td>
<td>Medulla edema</td>
</tr>
<tr>
<td>19 Day 2, 12 hours after 2nd Injection</td>
<td>No lesion</td>
</tr>
<tr>
<td>20 Day 2, just after 2nd Injection</td>
<td>Pons infarction</td>
</tr>
<tr>
<td>21 Day 3, 20 hours after 2nd Injection</td>
<td>No lesion</td>
</tr>
<tr>
<td>22 Day 2, 1 hour after 2nd Injection</td>
<td>Pons infarction</td>
</tr>
<tr>
<td>23 Day 2, just after 2nd Injection</td>
<td>Right hippocampus cortex infarction</td>
</tr>
</tbody>
</table>
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FIG 2. Angiograms taken on day 0 and day 3 of rabbit 13. SAH indicates subarachnoid hemorrhage.

exposed by dissecting the midline portion of the muscle layers and sufficiently abraded to be so thin that the cisterna magna could be identified through the membrane.

The first SAH was induced by injecting 0.9 mL/kg arterial blood into the cisterna magna. The arterial blood was drawn from the auricular artery. Two days later, 0.5 mL/kg of 1.2 mmol/L OxyHb was injected into the cistern to induce a second SAH. Each injection was done over a period of 10 seconds. The animals were then positioned with their head down for 10 minutes. OxyHb was prepared according to the method described by Okada et al. 20

Cerebral Angiography

Right or left CCA was exposed in a sterile manner through a median incision in the cervical region. A 19-gauge polyethylene catheter was inserted into the CCA and retrogradely advanced into the aortic arch. Anterograde angiography of the vertebrobasilar arterial system was performed by injecting 5 mL ioxaglic acid 320 mg I/mL into the aorta.

Baseline angiography was conducted before the initial injection of arterial blood into the cisterna magna, ie, at 2 weeks after the CCA ligation. The following angiography was performed after the second injection of OxyHb (day 3 or 4).

Measurement of Local CBF

Just after the second angiography in 14 rabbits of the SAH group, local CBF in bilateral parietal lobe cortex was measured by using the inhalation hydrogen clearance method. Tracheostomy was performed, and the animals were placed under controlled ventilation. Oxygen was administered to keep arterial Pco₂ and Po₂ levels at 35 to 40 mm Hg and 150 to 250 mm Hg, respectively.

Histological Examination

The brains were removed from the cranium and fixed in 10% formalin solution. The tissues were processed and embedded in paraffin, and 3-µm-thick sections were cut and stained with hematoxylin and eosin. The presence, location, and size of ischemic brain damage were observed. This study was also done with the 5 rabbits of the CCA ligation group.

Data Analysis

The diameter of the basilar artery was measured on each film at three points using a magnifier. The average value of the three points was obtained, and the percentage of reduction of the basilar artery before (baseline) and after induced SAH was investigated. The results of CBF measurement in the SAH group were compared with those of the untreated and CCA ligation groups and those of each neurological deficit grade group within the SAH group. For these variables, statistical evaluation was performed by ANOVA within groups. A value of P<.05 was considered to be statistically significant.

Results

Neurological Symptoms

The 8 rabbits in the SAH group showed severe symptoms such as quadriplegia or respiratory impairment after OxyHb injection and died without recovery. The remaining 15 rabbits showed progressive aggravation of neurological symptoms. Symptom grade of these

![Graph showing neurological symptom and basilar artery constriction.](http://stroke.ahajournals.org/)

Fig 3. Bar graph of neurological symptom and basilar artery constriction. There was no significant correlation between neurological grade and the constriction ratio.
15 rabbits was defined just before angiography on day 4 or at the time point when neurological symptoms progressed to grade 4, as shown in Table 3. Eight of 15 rabbits showed significant neurological symptoms of grade 3 or 4, and quadriplegia was observed as a frequent symptom. The other 7 rabbits showed minimum symptoms of grade 2. Grade 3 or 4 was considered to represent the presence of significant neurological symptoms. Angiographic study and measurement of CBF were completed in only 12 or 14 rabbits, respectively. Some rabbits that were in serious condition before study died or showed severe hypotension and were excluded from this study to ensure reliable data.

Cerebral Angiography

Angiograms taken on day 0 and day 3 of a rabbit with grade 3 symptoms are shown in Fig 2. Diffuse constriction of the basilar artery and the circle of Willis was observed after induced SAH. The mean±SD constriction rate of the basilar artery in each group of grades 2, 3, and 4 were 24.8±7.7, 26.3±12.6, and 40.8±10.0, respectively. This result was inconsistent, and there was no significant correlation between neurological grade and severity of the constriction ratio (P>.05) (Fig 3).

Local Cerebral Blood Flow

The mean±SD local CBF (in mL · min⁻¹ · 100 g brain⁻¹) was 41.4±1.7 in untreated rabbits without CCA ligation, 39.6±1.7 in those with CCA ligation, and 33.3±5.7 in the SAH group. In the SAH group, the mean±SD (in mL · min⁻¹ · 100 g brain⁻¹) was 38.6±2.8 in grade 2, 31.0±1.8 in grade 3, and 27.6±4.4 in grade 4.

The level of local CBF was significantly lower in subjects with apparent neurological symptoms (grade 3 or 4) than in untreated, CCA ligation, or grade 2 symptom rabbits (P<.01) (Fig 4).

Histology

In light microscopic examination, histological lesions were seen in 10 of 23 rabbits in the SAH group (43.5%). On the other hand, no lesion was observed in 5 rabbits of the CCA ligation group. Among the SAH group, an ischemic lesion was seen in 2 of 7 rabbits with grade 2 symptoms; the lesion of 1 rabbit was delayed neuronal death of the cerebral cortex and that of the other was infarction of the right hippocampus. No lesion was seen in rabbits with grade 3 symptoms. An ischemic lesion was seen in 2 of 4 rabbits with grade 4 symptoms; one lesion was infarction of the pons and the other was infarction of the left hippocampus (Fig 5). Furthermore, lesions were observed in 6 of 8 dead rabbits. There were 2 cases of edema of the medulla oblongata alone, 1 of edema of the medulla and cerebral cortex, 2 of infarction of the pons alone, and 1 that exhibited infarction of the pons, cerebral cortex, and right hippocampus.

All the results are summarized in Table 3.

Discussion

Endo et al 18,20 have developed and evaluated a rabbit model of vasospasm in which neurological symptoms are used as an indicator. In that model, CCA is bilaterally tied to establish the blood supply route mainly from the vertebrobasilar arterial system into the entire brain. Expected cerebral ischemia is considered to be caused by vasospasm of the basilar artery and the entire arterial circle of Willis. However, the problems with that reported model are that ischemic lesions do not appear in histological examination18 as sometimes seen in clinical patients, and that pathophysiological study is lacking. In this study, we used OxyHb for the second injection instead of arterial blood and added CBF measurement study.

Oxyhemoglobin is thought to make some contribution to the development of vasospasm after SAH, even if oxyhemoglobin itself is not an etiologic substance. As indicated in other rabbit models, it is thought that vasospasm becomes highly intense in conjunction with degeneration of the hematoma 2 to 3 days after the arterial blood floods into the subarachnoid space and that the level of oxyhemoglobin in the subarachnoid space increases at this time. Our new experimental model assumes that additional injection of OxyHb in such a state should cause the more intense...
contraction of the blood vessels associated with severe neurological symptoms and ischemic changes.

The incidence of neurological deficits was as frequent as in a two-hemorrhage model, however, severe deficits or death tended to be more remarkable in this new model. Furthermore, ischemic brain lesions were observed in 10 of the 23 rabbits, whereas few lesions were seen in previously reported models. Edema is not specific to ischemic change; however, the edema found in the 3 dead rabbits could be a kind of ischemic lesion caused by radical arterial spasm brought on by OxyHb injection. From these results, it is suggested that this presented SAH model can be more symptomatic.

The CBF was significantly lower in the SAH group than the untreated or CCA ligation group; it also differed significantly between rabbits with neurological symptoms of grade 3 or grade 4 and those with grade 2. As the CBF is measured at parietal cortex, it might not represent entire brain function. However, considering the result that basilar artery diameter was not correlated with neurological symptoms, the CBF value could be a more reliable indicator of cerebral vasospasm than angiography. Generally, if regional CBF is below 18 to 20 mL/min per 100 g brain, neurological deficit or infarction is likely to ensue. The CBF value in this study is about 25 to 30 mL/min per 100 g brain with apparent neurological deficit of grade 3 or 4, and 35 to 40 mL/min per 100 g brain with minimum deficit of grade 2. This CBF value with apparent deficit might be at a critical level, and if it could be reduced to below 20, a more stable model of cerebral vasospasm could be achieved with neurological deficit and organic ischemic lesion.

References

Editorial Comment

Although with modern management (which combines hemodilution, hypervolemia, and hypertension) vasospasm after subarachnoid hemorrhage is no longer a complication exacting a toll as high as in the past, there are still many frustrating experiences where effective pharmacological treatment or prevention would have been most welcome. Thus, there is still a need for a model that should have the following features: it should not be too expensive, to allow performance of the rather large number of experiments necessary to prove effectiveness of any therapy; it should incorporate angiographic demonstration of vasospasm and measurements of cerebral blood flow to gauge hypoperfusion so that the effect on both the conducting and resistance vessels can be assessed; it should incorporate the demonstration of histological changes in the brain tissue; and it should display clinical manifestations of vasospasm. Of all the models used to study vasospasm in experimental animals, the one described in the article by Otsuji et al above comes closest to fulfilling these desirable criteria.

If the results can be replicated in other laboratories, this model will be very useful in testing therapies that are clinically relevant to human vasospasm.

J. Paul Muizelaar, MD, PhD, Guest Editor
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Richmond, Va
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