Impaired capillary perfusion and brain edema following experimental subarachnoid hemorrhage: a morphometric study


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To evaluate microcirculatory disturbance and cerebral edema associated with subarachnoid hemorrhage (SAH), both stereological morphometry on the intraparenchymal capillary network and microgravimetry were performed on a rabbit SAH model. Autologous arterial blood (5 ml) was injected into the cisterna magna, and the animals were sacrificed at intervals of 6 hours, 1 day, 2 days, or 6 days after SAH. Capillaries in the piriform cortex, parasagittal cortex, and ventral brain stem of the midline-hemisectioned brain were injected with Evans blue dye 1 minute before sacrifice, and were planimetrically evaluated under a fluorescence microscope connected to an image analysis system. Stereological and morphological parameters including the volume density, surface density, numerical density, minimum intercapillary distance, and the diameter of Evans blue-perfused capillaries were also computed.

In the piriform cortex and ventral brain stem, the volume and surface densities were significantly reduced and the minimum intercapillary distance was significantly increased 1 to 2 days after SAH. In the parasagittal cortex far from the cisternal clot, changes in the parameters were minimal. Cerebral blood volume (CBV) in the normal condition and edema formation associated with SAH were studied by the microgravimetric technique. The mean CBV in the parasagittal cortex, piriform cortex, and brain stem was 6.9%, 6.8%, and 5.6%, respectively. Following SAH, specific gravity in the piriform cortex and the ventral brain stem of the other side of the hemisectioned brain was significantly decreased at 1 to 2 days, showing a change parallel to that of the stereological parameters. The results obtained from the morphometric technique indicated the occurrence of impaired capillary perfusion and reduced capillary blood volume following SAH, while microgravimetry suggested the formation of brain edema during this period. These changes in the intraparenchymal vessels may play an important role in the pathophysiology of SAH.

KEY WORDS - microcirculation • capillary • morphometry • subarachnoid hemorrhage • specific gravity • cerebral blood volume • rabbit
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Use of Laboratory Animals and was further approved by the University of Virginia Animal Research Committee. Thirty-seven male New Zealand White rabbits weighing 2.5 to 3.5 kg each were anesthetized with an intramuscular injection of a mixture of xylazine (6 mg/kg) and ketamine hydrochloride (30 mg/kg), and were intubated and mechanically ventilated with a respirator.* Skeletal muscle paralysis was achieved with pancuronium bromide (0.1 mg/kg). Teflon catheters (No. 24) were inserted percutaneously into a marginal ear vein and auricular artery for the administration of drugs, monitoring of mean systemic arterial pressure (SAP), and sampling of arterial blood. Expired CO₂ was monitored continuously and arterial blood gases were measured before the induction of SAH. The animals were placed in a 30° head-down position, and a No. 23 butterfly needle was inserted percutaneously into the cisterna magna in an aseptic manner. Experimental SAH was induced by injecting 5 ml of nonheparinized fresh autologous arterial blood over a period of 30 seconds. Control animals and saline-perfused control animals did not receive any intrathecal injection. After induction of SAH, the animals were allowed to awaken and were returned to their cages. The general and neurological conditions of the animals were examined daily.

Tissue Preparation

Under the same anesthetic procedure and physiological monitoring, the animals were sacrificed by an intravenous injection of saturated KCl solution at intervals of 6 hours, 1 day, 2 days, or 6 days after SAH. Prior to sacrifice, physiological parameters such as arterial pH, pO₂, pCO₂, base excess, hematocrit, and plasma osmolality were measured. Except for the saline-perfused control animals, 2% Evans blue dye in physiological saline was injected into animals at a dose of 2 ml/kg, and allowed to circulate for 1 minute. The brain was rapidly removed and cut sagitally on the midline. The left half of the brain was immediately immersed into cold 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4), with sucrose at a final concentration of 8%. For fluorescence microscopy, frozen sections 8 μm thick were prepared from the vertical plane of the piriform cortex (pars caudalis), parasagittal cortex, and ventral brain stem (pons). The right half of the brain was kept in an ice-cold moist chamber and used to measure SG.

Fluorescence Microscopy and Morphometric Analysis

The capillary network perfused with Evans blue dye was examined under a fluorescence microscope** connected to a computer-assisted image analysis system.† For each animal, data from five microslides randomly selected from 25 serial sections were compiled. Excitation light was transmitted to the sections via an excitation filter and a beam splitter. Emitted light was passed through a barrier filter. Perfused capillaries from each microslide were traced by a digitizer on the five different measuring fields in the superficial area (from the pial surface to 200 μm in depth; tentatively termed “Site 1” in the section) and the adjoining area (200 to 400 μm in depth, “Site 2” in the section) of each brain region as previously stated. To evaluate changes in microcirculation after SAH, stereological parameters** including volume density (cu mm/cu mm), surface density (sq mm/cu mm), and numerical density (no./cu mm) of Evans blue-filled capillaries were computed by area-perimetry analysis, using a Zeiss Videoplan system equipped with a stereology program. The minimum intercapillary distance (μm³) and minimum capillary diameter (μm) were also measured in the same fields. Vessels with minimum diameters of more than 10 μm were excluded from the data. An average of the pooled data for each parameter was used for analysis. Correlations between the morphometric parameters and SG were examined using the averaged values for each morphometric parameter between Sites 1 and 2 in each experimental animal.

Microgravimetry

To estimate edema formation following SAH, the SG of the brain samples (10 to 20 mg each) obtained from the piriform cortex, parasagittal cortex, and ventral brain stem of the contralateral side were measured by the microgravimetric technique reported by Marmarou, et al. The mean of the SG values from triplicate samples was used to represent the SG value of each brain region. In order to evaluate the influence of cerebral blood volume (CBV)** on brain SG, six animals without SAH were subjected to transcardial perfusion with warmed physiological saline. The SG values of the saline-perfused brain thus obtained were used to calculate CBV and the SG of the bloodless brain in the control animals by the equation suggested by Shigeno, et al.** as follows: CBV = (A - B)/(a - b) × 100%, and SG of the bloodless brain = (aB - bA)/(a - b) - (A - B), where A is the SG obtained from control animals, B is the SG of brain from saline-perfused animals, a is the SG of the blood, and b is the SG of physiological saline.

Statistical Evaluation

Analysis of variance and the Tukey test were used to evaluate statistical significance. Results were expressed as means ± standard deviation. Differences of distributions were evaluated by the Kormogorov-Smirnov two-sample test. A probability value of less than 0.05 (two-tailed hypothesis) was considered significant. The Pearson correlation coefficient was used to analyze the
TABLE 1
Physiological parameters at time of sacrifice

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>No. of Animals</th>
<th>SAP (mm Hg)</th>
<th>pH</th>
<th>pO₂ (mm Hg)</th>
<th>pCO₂ (mm Hg)</th>
<th>Hematocrit (%)</th>
<th>Plasma Osmolality (mOsm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7</td>
<td>87 ± 15</td>
<td>7.5 ± 0.05</td>
<td>133 ± 29</td>
<td>36 ± 3.1</td>
<td>30 ± 2.8</td>
<td>307 ± 4.3</td>
</tr>
<tr>
<td>control (saline-perfused)</td>
<td>6</td>
<td>86 ± 16</td>
<td>7.4 ± 0.10</td>
<td>129 ± 36</td>
<td>36 ± 9.4</td>
<td>31 ± 2.1</td>
<td>308 ± 4.2</td>
</tr>
<tr>
<td>6 hrs</td>
<td>6</td>
<td>79 ± 16</td>
<td>7.4 ± 0.07</td>
<td>135 ± 31</td>
<td>35 ± 4.9</td>
<td>31 ± 4.3</td>
<td>313 ± 9.5</td>
</tr>
<tr>
<td>1 day</td>
<td>6</td>
<td>64 ± 20</td>
<td>7.4 ± 0.06</td>
<td>130 ± 35</td>
<td>35 ± 5.5</td>
<td>31 ± 1.5</td>
<td>315 ± 3.8</td>
</tr>
<tr>
<td>2 days</td>
<td>6</td>
<td>72 ± 6</td>
<td>7.4 ± 0.10</td>
<td>139 ± 21</td>
<td>35 ± 8.1</td>
<td>29 ± 1.9</td>
<td>316 ± 3.8</td>
</tr>
<tr>
<td>6 days</td>
<td>6</td>
<td>79 ± 18</td>
<td>7.4 ± 0.05</td>
<td>139 ± 33</td>
<td>40 ± 9.0</td>
<td>28 ± 1.6</td>
<td>317 ± 8.4</td>
</tr>
</tbody>
</table>

* Data are expressed as means ± standard deviations. SAP = systemic arterial pressure.

TABLE 2
Morphometric parameters in control animals

<table>
<thead>
<tr>
<th>Morphometric Parameters</th>
<th>Piniform Cortex</th>
<th>Parasagittal Cortex</th>
<th>Ventral Brain Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site 1</td>
<td>Site 2</td>
<td>Site 1</td>
</tr>
<tr>
<td>Volume density (cu mm/cu mm)</td>
<td>0.031 ± 0.010</td>
<td>0.029 ± 0.009</td>
<td>0.034 ± 0.013</td>
</tr>
<tr>
<td>Surface density (sq mm/cu mm)</td>
<td>0.010 ± 0.003</td>
<td>0.009 ± 0.003</td>
<td>0.010 ± 0.003</td>
</tr>
<tr>
<td>Numerical density (no./cu mm)</td>
<td>0.025 ± 0.005</td>
<td>0.023 ± 0.007</td>
<td>0.023 ± 0.003</td>
</tr>
<tr>
<td>Minimum intercapillary distance (μm)</td>
<td>4.8 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Capillary diameter (μm)</td>
<td>47.5 ± 3.5</td>
<td>44.9 ± 3.8</td>
<td>65.0 ± 5.5</td>
</tr>
</tbody>
</table>

* Values are expressed as means ± standard deviations.

Fig. 1. Photomicrograph showing intravascular fluorescence of Evans blue dye-perfused capillaries in the piriform cortex of the control rabbit brain. Bar: 50 μm.

relationships between SG and morphometric parameters.

Results
Physiological Parameters and General Observations

Mean SAP and blood gas analysis were not significantly different between the rabbit groups before SAH. Immediately after SAH, mean SAP showed increases in the range of 12 to 97 mm Hg in all SAH groups. The animals awoke promptly from anesthesia and showed no apparent neurological abnormalities. The physiological parameters monitored prior to sacrifice are shown in Table 1. Although mean SAP tended to decrease in animals sacrificed on Day 1 or 2, there were no significant differences between experimental groups. Three animals died between 1 and 2 days after SAH. In those animals, the brain was macroscopically swollen and a large cisternal clot was observed. These animals were excluded from the experimental groups. Animals sacrificed 6 hours to 2 days after SAH had thick subarachnoid clots on the ventral surface of the brain stem and the base of the piriform cortex. Six days after SAH, the cisternal clots had been partly absorbed, with remnants on both sides of the basilar artery and traces at the base of the hemispheres.

Morphometric Evaluation

Fluorescence microscopic views of the Evans blue dye-perfused capillaries in the piriform cortex of a control animal are shown in Fig. 1. On examination by fluorescence microscopy, SAH animals tended to show a decrease in the area occupied by Evans blue-perfused capillaries and showed discontinuities when compared with the control animals. Since extravasations or perivascular globules of Evans blue dye around the capillaries were not observed during this period of circulation, there were no major difficulties in performing morphometric evaluations of the perfused capillaries.

The average values of morphometric parameters obtained from control animals are shown in Table 2. Changes in the morphometric parameters in each brain region following SAH are indicated in Fig. 2. One to 2 days after experimental SAH, volume density and surface density decreased, and the minimum intercapillary
distance increased significantly in the superficial region of the piriform cortex (Site 1 in the section, Fig. 2 upper). However, average values for numerical density in the SAH animals did not show any significant difference compared with those of the control group, although values tended to decrease on Days 1 to 2 after SAH. The changes in minimum intercapillary distance were the same at Site 2 of the piriform cortex as those at Site 1. Among the other morphometric parameters examined, only volume density decreased significantly in animals sacrificed on Day 1.

In the parasagittal cortex, a decrease in volume density and an increase in minimum intercapillary distance were observed only at Site 1 of Day 1 animals, but the changes were not remarkable. At Site 2, there were no significant differences in any of the morphometric parameters (Fig. 2 center).

In the ventral brain stem, stereological parameters showed relatively small values and the minimum intercapillary distance was larger than in the cerebral hemisphere (Fig. 2 lower). Significant differences were observed in volume density, surface density, and minimum intercapillary distance at Site 1 of Day 1 animals. At Site 2, the values of the morphometric parameters were similar to those of the hemisphere, which is probably due to the existence of gray matter in the brain stem. The changes in parameters after SAH were the same as at Site 1.

Representative frequency distribution histograms of minimum intercapillary distance are shown in Fig. 3. The SAH exerted a significant influence on the shape of the distribution of intercapillary distances (p < 0.001). The average values of capillary diameter ranged from 4.50 μm (at Site 1 of the piriform cortex in Day 1 animals) to 5.72 μm (at Site 1 of the ventral brain stem in Day 6 animals) and showed no significant differences between experimental groups.

Microgravimetry
Average values of SG, calculated CBV, and tissue...
SG from the control and physiological saline-perfused control animals are given in Table 3. The average SG value of rabbit blood was 1.0507 ± 0.0022. For the SG value of physiological saline, 1.0064 was employed. The lowest possible SG values calculated from 99% confidence limits of the mean values from control animals are given in Table 3. 31 The average SG value of physiological saline-perfused group was 1.0411 ± 0.0001. The lowest possible SG values calculated from 99% confidence limits of the control groups are also given in Table 3.

The changes in SG values after SAH are shown in Fig. 4. Specific gravity in the piriform cortex and ventral brain stem were significantly reduced in animals sacrificed 1 or 2 days after SAH. In the parasagittal cortex, SG values showed no significant change during the experimental period.

Correlations Between Morphometric Parameters and Specific Gravity

A significant linear correlation between SG and minimum intercapillary distance was observed (r = -0.471, p < 0.001: Fig. 5). Correlations between SG and stereological parameters (volume density, surface density, and numerical density) were not apparent, although they were statistically significant (r = -0.303, r = 0.252, r = 0.293, respectively; p < 0.01). The relationship between SG and capillary diameter was not significant (r = -0.02, p = 0.79).

Discussion

Morphometric Evaluation and Experimental SAH

Quantitative morphometric evaluations of the capillary network in the brain have been made with regard to physiological and pathological conditions such as normal, asphyxiated brain, hemorrhage, development and aging, ischemia, cerebral swelling, and SAH. Weiss, et al., and Im Dahl and Hoss- mann evaluated dynamic microcirculatory changes by stereological morphometry using intravital dye tracers. To assess the perfusion changes in intraparenchymal capillary networks after experimental SAH, we adopted a similar approach using Evans blue dye as a tracer, which is readily fixed by aldehydes. The values of the stereological parameters obtained in the present study were in close agreement with those of the perfused rat capillaries reported by Weiss, et al., despite the difference in procedures.

Following SAH, volume density decreased significantly in the piriform cortex and the ventral brain stem especially in the superficial region, which was in direct contact with the cisternal clot. The shape of the capillaries often had an interrupted appearance, which may have been caused by microcirculatory stasis, possibly leading to the changes in morphometric parameters (the decrease in volume density and surface density and the increase in minimum intercapillary distance). Among the stereological parameters used, the change in numerical density was found to be nonsignificant, although the average values tended to decrease in Day 1 animals after SAH. This dissociation between parameters was seen in another report, although it might be partly due to an artifact resulting from the thickness of the sections. In the parasagittal cortex, changes in the morphometric parameters were far less remarkable than in the other regions examined.

Although cerebrovascular spasm is characterized by a prolonged consistent constriction of the major cerebral arteries, the condition of the radiologically invisible intraparenchymal vessels during the period of vasospasm is unclear. Grubb, et al., reported increased CBV in poor-grade SAH patients. Their reevaluation by positron emission study has shown similar results. Using a photoelectric sensor, Kuyama, et al., observed an initial decrease and subsequent increase in CBV during the acute stage of SAH.

In contrast, however, several morphological studies have demonstrated general constriction or impaired perfusion of the intraparenchymal vessels following experimental SAH. Using the morphometric approach, Hart and Wiernsperger, et al., have quantitatively demonstrated the constriction of intraparenchymal vessels following cisternal injection of blood or plasma. Asano and Sano and Nagai, et al., also reported microcirculatory disturbances in dogs following SAH using carbon black perfusion in the acute and prolonged stages. The present results on the morphological change in the microvascular network and its time course were qualitatively similar to those of Nagai, et al. Interestingly, studies based on the morphological approach...
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Fig. 4. Block graph showing changes in specific gravity in each experimental group. Values are expressed as means ± standard deviations. * p < 0.05, ** p < 0.01.

suggest constriction of the intraparenchymal vessels, while those based on physiological techniques indicate vasodilation of the vessels. Of course, in the morphological approach, it is obviously impossible to serially evaluate in vivo changes in microcirculation. The effects of the fixative and postmortem changes should also be taken into account. However, studies using radioactive tracers or a photoelectric sensor may be influenced by red blood cell stasis and plasma skimming. Clearly, these studies cannot be compared in the same manner. Discrepancies in findings may result from limitations inherent to the technique adopted or the observation period. Since it is important to delineate the pathophysiological changes following SAH, further integrated studies are necessary.

Estimation of Edema Formation and CBV by Microgravimetry

In the present study, significant reduction of cortical specific gravity was demonstrated 1 to 2 days after SAH. Brain edema following experimental SAH has been reported to occur in the very early (1 to 6 hours) period and relatively subacute stage, as late as 48 hours. Generally, in the punctured artery hemorrhage model, edema tends to occur in the very early stage, while in cisternal injection models, including our model, it is most remarkable around 24 hours post-SAH. In the present experimental design, we did not try to differentiate the effects of lysed clots from other pathological factors such as changes in intracranial pressure or cerebral blood flow using blood-free substances. Although the above reports and our findings suggest that the cause of edema may be multifactorial and occur at various intervals following SAH, future studies should include the intrathecal injection of a non-vasoactive fluid not rapidly absorbed to determine the exact time course of edema formation.

Regarding changes in SG, the effects of SAH appear to be less than those in regional cerebral ischemia. Furthermore, there is controversy as to whether SG is influenced by changes in CBV, although microgravimetry is a highly sensitive tool for the estimation of tissue water content. The calculated CBV in normal rabbits was similar to that in the cat reported by Shigeno, et al. However, as suggested by Picozzi, et al., the SG values of the piriform cortex and ventral brain stem, especially in Day 1 animals, were further reduced compared with SG values of completely bloodless tissue in corresponding sites. On the other hand, morphometry indicated that capillary blood volume was significantly reduced around Day 1 after SAH and a significant correlation between minimum intercapillary distance and SG has been documented. From the above results, although microgravimetric estimation of the contribution of capillary volume to CBV in the pathological condition has not been performed, it is conceivable that both factors (the decrease in CBV and increase in water content) resulted in the reduction of SG after SAH.

Factors Contributing to Impaired Capillary Perfusion

Our results showed that impaired capillary perfusion, decreased CBV, and brain edema coexist in the course of experimental SAH. This explains the persistent disturbance of cerebral circulation found in the course of clinical SAH. Since the capillaries are regarded as a mechanically rigid system, this circulatory disturbance was probably brought about by increased vascular resistance in the arterial tree proximal to the capillaries. Fukasawa and Heistad, et al., have pointed out that large extraparenchymal arteries and penetrating arteries contribute to cerebrovascular resistance as well as to the resistance of arterioles. The prolonged spasm of the major artery in the present model lasts from 1 to 3 days after SAH. Impaired capillary perfusion associated with a reduction in SG was most remarkable in the region where the cisternal clot was in direct contact with the brain surface. The arteries on or near the pial surface are reportedly influenced by the cisternal clot penetrating into the Virchow-Robin space. The formation of ischemic brain edema...
would have further compressed the capillaries. Such a pathophysiological process can lead to a vicious circle and contribute to the origin of symptomatic vasospasm in the clinical situation. Although the etiology of vasospasm and its proper management are not yet established, the present findings suggest the effectiveness of intravascular volume expansion and induced hypertension therapy.

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