Cerebral vasospasm is a major factor contributing to poor outcome in patients suffering from SAH. The main features of cerebral vasospasm are delayed onset, prolonged contraction of major cerebral arteries, and resistance to most known vasodilators. Nonetheless, the major arterial vasospasm that can be illustrated angiographically does not always correlate with cerebral ischemic symptoms or CBF. Some medications, such as nimodipine, fail to prevent or reverse angiographic vasospasm, but do improve clinical outcomes of patients; other therapies, such as angioplasty, reverse angiographic vasospasm, but fail to improve neurological deficits.

This apparent paradox has led some investigators to hypothesize that the cerebral microcirculation is affected by cerebral vasospasm. The intravascular components or intraparenchymal microcirculatory changes must be considered as possible contributory causes of cerebral ischemia. Indeed, we have recently found that apoptotic changes occurred in cultured endothelial cells and in vasoactive arteries, and that vasospasm extended into small penetrating arteries in the extraparenchymal space. Nonetheless, the cerebral microcirculatory changes inside the parenchyma that have been observed during cerebral vasospasm after aneurysmal SAH are still controversial. In this study, we used an established canine double-hemorrhage model to evaluate intraparenchymal microvasculature for the presence of vasospasm and to examine the surrounding neuropil for cellular damage in the pons.

Materials and Methods
The protocol for this study was evaluated and approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee.

Experimental SAH Model
Twelve adult mongrel dogs of either sex, each weighing between 18 and 24 kg, were used for this study. Nine animals were randomly selected for the double-hemorrhage model and three were selected for controls. Anesthesia was induced in the animals by administration of thiopental (10 mg/kg) and mechanical ventilation was maintained during the experiments. A heating blanket maintained the animals’ body temperature at 37°C. The mean arterial blood pressure and blood gases were monitored using a cather inserted...
into the femoral artery and maintained within normal ranges. Experimental SAH was induced according to the method of Varsos, et al., as described by Tibbs, et al.,1 previously. Cerebral angiography was performed with the aid of a fluoroscope (Exposcop 7000; Ziehm International Medical Systems, Riverside, CA). After a baseline vertebrobasilar angiogram had been obtained, the cisterna magna was punctured transcutaneously, and 0.4 ml/kg of cerebrospinal fluid was withdrawn. An equivalent amount of arterial blood was withdrawn from the femoral artery and immediately and slowly injected into the cisterna magna. The dogs were placed head down in a prone position and tilted 20° for 20 minutes to permit pooling of the blood around the BA. After 2 days, the blood injection procedure was repeated without angiography. Control animals received blood injections but no angiography. On Day 7 all animals were perfused with a cocktail of 1% paraformaldehyde and 1% glutaraldehyde, followed by brain removal and postfixation of the pons. The perfusion pressure ranged from 70 to 80 mm Hg (with a flow rate of 43 to 45 ml/minute). The perfusion fixative was delivered using a peristaltic pump (Master flex Model 7; Cole-Parmer Instrument Co., Vernon Hills, IL).

Measuring the Diameter of the Arteries

Artery diameters were measured on magnified angiograms in a double-blinded fashion, as described previously. A penny was placed on the dog’s chin during the angiography session, and, using the size of this coin as a standard, the values of all artery diameters were adjusted. Two researchers independently measured the diameters of the arteries on the magnified angiograms at three points: the distal, central, and proximal portions of the BA. The mean of these three measurements was calculated to yield the diameter of the artery. The caliber of the BA on Day 7 was calculated as the percentage of the mean BA diameter determined on Day 0 in each dog.

Histological Study

Coronal sections, 2 mm thick, were excised from the midpons of both control animals and dogs subjected to SAH. The sections were dehydrated, embedded in paraffin, and sectioned axially from the ventral surface at a thickness of 10 μm and a depth of 8 mm. The sections were stained with either cresyl violet or hematoxylin and eosin. Cresyl violet- and hematoxylin and eosin-stained sections were used to evaluate the morphological characteristics of neurons and the neuropil, and the morphometry of vessels. Morphological studies of neuronal cells performed at a structural level accessible to light microscopy were done by surveying slides and grids, respectively. Cells were observed, counted, and graded as normal or abnormal. Several serial sections representing tissue depths ranging from the pontine surface to 5 mm below the surface were examined in both control dogs and dogs with SAH.

Vessel Morphometric Studies

Vessel morphometry, modified from a method reported previously, was performed on the hematoxylin and eosin-stained sections by using a digital imaging system.10 All parenchymal vessels, including arterioles and venules, were measured to determine luminal area and perimeter, and wall thickness and area. We randomly selected arterioles and venules from sections obtained from the ventricle at a thickness of 10 μm and a depth of 50 μm to 5 mm. Vessels were captured and saved as digital images by using the digital imaging system. Special attention was paid to recording the depth of the tissue section to provide the best comparison between control dogs and dogs with SAH.

After a review of the raw data, morphometric measurements of arterioles were generated from 96 slides (eight slides/dog) with a mean of seven to 10 vessel cross-sections/slide. Morphometric measurements for venules were generated from 120 slides (approximately 10 slides/dog) with a mean of seven to 10 vessel cross-sections/slide. Twelve dogs were used in the study, nine dogs subjected to experimental SAH and three control dogs.

Morphometric measurements of vessels were obtained using a light microscope, a digital camera, and a computer equipped with morphometric software (Metamorph; Universal Imaging Corp., Downingtown, PA). Each vessel (arteriole and venule) selected for measurement was digitally captured and measured using the tools provided within the software. The diameters of the vessels were measured by tracing the entire luminal surface of the intima with the aid of a mouse-guided cursor. Measurements of the lumen, but not the diameter of the lumen, were recorded without interpretation of luminal diameter when oval or irregularly shaped vessels are measured. Vessel wall thickness was measured using a software bar tool to transverse the wall of the vessel at four different point locations (90° across the arterial wall from each other) and was expressed as a mean of the four points. The measurement of wall thickness was extended from the luminal surface of the intima to the outer limits of the media, not to include the adventitia. For vessel wall thickness measurements, a mouse-guided cursor was used to outline the external and internal walls of the vessel, respectively. We calculated the areas of the lumen and wall by using imaging analysis software (Metamorph) after we had obtained the measurements of both internal and external perimeters. All measurements were standardized with respect to resolution and magnification by using the calibration feature of the software.

Morphological Studies of Cells

The neurons of control animals and dogs with SAH were evaluated morphologically. Slides were scanned under light microscopy at a low magnification (× 200) to locate cells and at a higher magnification (× 400) to evaluate their grades. Cells were graded according to size (swelling or shrinkage), appearance, and the presence of vacuolation in the neuropil. Cells were observed at the light microscope level for changes in cell size (swelling or shrinkage) and the presence of vacuolation. Cell counts and gradings were performed using the following procedure: each slide was scanned at a high power and the first 100 cells encountered on each slide were graded as normal (no apparent change in size of cells and absence of vacuolation) or abnormal (cells appearing to have swollen or shrunk in size and/or presence of vacuolation). Several slides containing serial sections representing tissue depths ranging from the pontine surface to 5 mm below it were examined in both control dogs and dogs with SAH. More than 2500 cells from both control dogs and dogs with SAH were graded.

Electron Microscopy Studies

Tissues from the midpons were prepared for electron microscopy processing, as described previously.10 Samples 1 mm thick and 4 mm in height were postfixed with osmium tetroxide, dehydrated in a graded series of acetone, embedded in epon-araldite epoxy resin, sectioned at 60 Å, and examined with the aid of a transmission electron microscope (Model 906; Leo, Thornwood, NY). Vessels were evaluated for dystrophic changes in smooth-muscle and endothelial cells, and for corrugation of the IEL.

Statistical Analysis

All data are expressed as the means ± SEMs. Statistical differences between the control and SAH groups were compared using the Student t-test. A probability value lower than 0.05 was considered statistically significant.

Results

Cerebral Vasospasm in the BAs

Severe vasospasm consistently occurred in the BAs of all dogs that received double blood injections. Figure 1 demonstrates the angiographic changes that occurred from Day 0 (lumen viewed as 100%) to Day 7 (peak vasospasm) in the diameters of BAs from four dogs with SAH. The mean value of the BA diameter on Day 7 (0.72 ± 0.02 mm) in these dogs was 47.39 ± 6.32% of the mean value measured on Day 0 (1.519 ± 0.05 mm, Day 0). Angiographic vasospasm in dogs with SAH was con-
firmed by histological examination, as shown in Fig. 2. In Fig. 2 upper, severe corrugation of the IEL and contraction of smooth-muscle cells are displayed in the BA excised from a dog with SAH. In Fig. 2 lower, a normal BA excised from a control dog is shown.

Vessel Morphometric Findings

Figure 3 schematically depicts the area and plane of sectioning of a dog brain for microvascular analysis. Slices were obtained from a coronal section from the pontine region; the slices were cut axially to reveal the microvessels and neuronal tissues in the pontine region. All studies represented by Figs. 4 through 8 are based on these cuts.

Morphometric examination by light microscopy and a digital imaging system revealed increased luminal area in parenchymal arterioles taken from dogs with SAH when compared with sections obtained in control dogs (p < 0.01, t-test) (Fig. 4A and B). There were no differences in the area or thickness of vessel walls in parenchymal arterioles between control dogs and dogs with SAH (p > 0.05, t-test) (Fig. 4C and D). In addition, we measured some parameters in the parenchymal venules in the same pontine region. No differences were noted in the luminal area, perimeter, or wall area in the parenchymal venules between control dogs and dogs with SAH (p > 0.05) (Fig. 5). The wall thickness in venules was not measured further.

Morphological Findings in the Vessels

The morphological features of parenchymal arterioles were examined with the aid of a transmission electron microscope. No dystrophic changes in smooth-muscle or endothelial cells and no corrugation of the IEL were noted in samples from the pontine region of dogs with SAH. A comparison of samples obtained from three control dogs and three dogs with SAH demonstrated that in both groups the arterioles were normal and no sign of vasospasm in these arterioles was evident (Fig. 6).
Vasospasm in microvessels

**Fig. 2.** Upper: Photomicrographs of a BA taken from a dog with SAH, showing the common characteristics of vasospasm: corrugation of the IEL and desquamation of the endothelial cells. Lower: Photomicrographs of a BA taken from a control dog lacking the corrugation and desquamation characteristics. H & E, original magnifications × 100 (left) and × 400 (right).

**Morphological Findings in the Cells**

A comparison of sample cells showed no sign of damage or stress when samples obtained from dogs with SAH were compared with samples obtained from control animals (Figs. 7 and 8). Neither hematoxylin and eosin nor cresyl violet staining revealed any obvious difference in neuron size or any evidence of chromatolytic change. In slides of sections obtained from control dogs or animals with SAH most nuclei were centrally located, there was abundant cytoplasm with equally distributed Nissl bodies, and no gliosis or gliocytic increases were revealed. All characteristics mentioned earlier are consistent with healthy neurons and neuropil (Figs. 7 and 8).

**Discussion**

To summarize the main observations of the current study, we can state the following: first, no vasospasm occurred in parenchymal arterioles, despite the fact that the BAs displayed severe vasospasm in dogs subjected to experimental SAH. Second, significant dilation was observed in parenchymal arterioles, probably because of compensation for reduced blood flow in the pontine region. Third, no neuronal ischemic damage was observed in the pontine region in this dog model. Thus, vasospasm does not extend into the parenchymal arterioles and does not cause neuronal damage in the pontine region in this experimental model of double-hemorrhage SAH in dogs.

In major cerebral arteries, vasoconstriction (vasospasm) is almost always accompanied by an increase in vessel wall thickness or wall area. Contraction of several layers of smooth-muscle cells increases the areas of the vessel wall and leads to narrowing of the lumen. In the present study, even though the diameters of the arterioles were increased, the wall thickness or wall area was not decreased as predicted from results obtained in major arteries. One possible reason is that only one or two layers of smooth-muscle cells exist in arterioles, compared with five or more layers of cells in major cerebral arteries. Contraction or relaxation of a few smooth-muscle cells in the arterioles might not have a strong impact on the thickness of a vessel wall. Dilation of arterioles, as observed in the present study, might stretch the matrix or other connective tissues in the vessel, which might reflex to a slightly larger area, as was measured in this study.

There are several ways of measuring vessel diameters or luminal areas. First, Hart measured the diameters and wall thicknesses of oval vessels by using a perpendicular line through the nonoval axis of the vessel. This method is now a widely accepted means to obtain a vessel diameter measurement, as exemplified in the literature. Ohkuma, et al., successfully used this method in the measurement of vessel diameter in cases in which vessels were cut in an angled plane of a section that showed an oval shape. Second, on the contrary, Fernie and Lamb measured the intimal area and defined artery size by the total length of the IEL. This method of vessel measurement is not affected by constriction or collapse of arteries because either may present in various planes of a section in histological slides. Third, Krasnoperov, et al., used a mathematical calculation to correct for errors in the measurement of microvessel perimeters and cross-sectional areas. In our effort to avoid the problems encountered in measuring the vessel diameter, we measured the vessel circumference, which is similar to the method described by Fernie, et al., to determine the size of the lumen. We believe that using the circumference to obtain vessel measurement, as used in the present study, is appropriate because circumference measurements avoid the problems encountered with diameter measurements when vessels are examined in an oval plane of section (diameter measurements are valid, however, when methods are used to correct for errors in situations of irregularly shaped vessels, as discussed earlier).

**Contradictory Phenomenon: Dilation of Arterioles During Vasospasm**

Physiologically, decreased cerebral perfusion pressure induces dilated peripheral intraparenchymal arterioles to compensate for decreased CBF. The intraparenchymal arterioles are known to be responsible for a significant portion of total cerebral vascular resistance. When vascular resistance is reduced under a low cerebral perfusion pressure, the CBF and brain oxygenation can be partially maintained.

Autoregulatory vasodilation of distal blood vessels can be detected in vivo by measuring an increase in parenchymal CBV. Various clinical investigators have suggested that intraparenchymal vessels are dilated in patients after SAH. Grubb, et al., demonstrated initially that CBV was increased in patients following SAH, suggesting that the cerebral microcirculation might, in fact, become vasodilated during severe cerebral vasospasm in the major arteries. Thus, cerebral vasospasm of large extraparenchymal arteries is accompanied by a massive dilation of the
FIG. 3. Schematic drawings of the dog brain showing the area and plane of sectioning. Slices were obtained from a coronal section from the pontine region that was cut axially. The measurement of arterioles and venules was made from the same section in each dog to minimize the possible bias that could result from measuring different sections.

FIG. 4. Bar graphs comparing differences in arterioles between control dogs and dogs with SAH. The results shown are from measurements of 96 arterioles. Bars represent mean measurements of arterioles and whiskers represent SEMs. *p < 0.05, t-test.
Vasospasm in microvessels

Intraparenchymal vessels. Martin, et al. reported that positron emission tomography studies also demonstrated increased CBV during cerebral vasospasm after SAH. Recently, Dietrich, et al., reported the importance of vasodilation propagation in regulating brain microcirculation. This phenomenon is initiated locally: vasodilation spreads upstream and downstream along a vessel. Kajita, et al., observed this propagated vasodilation after applying adenosine triphosphate, a proposed spasmogen, to cerebral arterioles. It seems likely that as BAs and extraparenchymal arteries in the subarachnoid space make contact with the blood clot, the intraparenchymal arterioles would only be affected by the propagated response.

This contradictory hemodynamic phenomenon might not relate to the diameter of the artery or arteriole, but it may depend rather on the location of these vessels. Indeed, small penetrating arteries can become vasospastic if they become saturated by the subarachnoid clot, which precipitates in the extraparenchymal spaces. Other investigators have previously observed similar occurrences when examining surface cerebral capillaries. Thus, one of the most important reasons that these arterioles are apparently spared from vasospasm probably relates to their particular locations inside the parenchyma, which do not allow blood components to infiltrate the parenchyma. The pia mater is located between the subarachnoid space and the perivascular spaces, and could effectively barricade red blood cells, preventing them from entering the perivascular spaces, even though the pia mater is permeable to a variety of substances. The pia mater barrier minimizes any direct effects of subarachnoid blood at the arteriolar level. The failure of the subarachnoid clot to extend along Virchow–Robin spaces surrounding the penetrating arterioles might explain the contradictory observations noted in this study: the severe vasospasm of the BAs was accompanied by dilation of the intraparenchymal or penetrating arterioles.

Controversial Results on Microcirculation in Vasospasm

Despite reports supporting our findings in this study, the majority of reports concerning microvasculature vasospasm have not always been consistent among themselves; the different regions of brain that have been evaluated and the models and methods that have been used might have caused these discrepancies. Table 1 summarizes some studies in which the microcirculation has been examined during cerebral vasospasm in humans and in animal models; it shows that no changes, increases, or decreases in the diameters of arterioles have been reported.

Different methods used to assess vasospasm in microvessels might yield contradictory results. Ohkuma, et al., reported that constriction of intraparenchymal arterioles occurs after SAH and that this might contribute to cerebral ischemia. If vasoactive substances derived from the subarachnoid clot could penetrate along the perivascular spaces, they might cause the perforating arteries to contract within the parenchyma. Multiple reasons probably explain the different observations noted in the study by Ohkuma, et al., and the present one. Although in both studies a double-hemorrhage model in dogs was used, we examined the brainstem region by mainly relying on histological methods, whereas Ohkuma, et al., used corrosion-casting methods and examined brain tissues from the anterior sylvian gyri. The fact that transmission electron microscopy was not used in the study by Ohkuma, et al., limited their observations to the light microscopic level, and the histological features of vasospasm could not be revealed. Nevertheless, if vasospasm occurs in the intraparenchymal arterioles and leads to cerebral ischemia and infarction, then clinical neurological deficits might also occur in animals, which does not seem to be the case in

Fig. 5. Bar graphs comparing differences in venules in control dogs and dogs with SAH. The results shown are from measurements of 120 venules. Bars represent mean measurements of venules and whiskers represent SEMs.
**TABLE 1**

<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>Model</th>
<th>Methods</th>
<th>Results</th>
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<td>Herz, et al., 1975</td>
<td>guinea pig pial microvessels &amp; SAH</td>
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<td>Grubb, et al., 1977</td>
<td>human clinical study</td>
<td>measurements of rCBV, rCBF, &amp; rCMRO₂ in patients w/ SAH</td>
<td>constriction of large extraparenchymal vessels accompanied by massive dilation of intraparenchymal vessels</td>
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<td>Hart, 1980</td>
<td>cat SAH induced by injection of autologous blood into cisterna magna</td>
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</tr>
<tr>
<td>Brandt, et al., 1981</td>
<td>exposure of human pial arterioles to various vasoactive agents including CSF from patients w/ SAH</td>
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<td>SAH in cats</td>
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<td>arterioles had a consistent vasoconstrictive response to CSF</td>
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<td>Vollmer, et al., 1992</td>
<td>rabbit SAH model</td>
<td>measurements of rCBF w/ autoradiography</td>
<td>SAH elevated ICP &amp; caused a transitory fall in cerebral perfusion pressure</td>
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<tr>
<td>Takayasu, et al., 1995</td>
<td>intracerebral arterioles exposed to oxyHb &amp; treated w/ vaso-pressin in vitro</td>
<td>measurements of vessel contractility of BAs &amp; penetrating arteries by using isometric tension</td>
<td>reactivity of intraparenchymal resistance vessels is not significantly altered after SAH</td>
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<td>Kajita, et al., 1996</td>
<td>penetrating arterioles exposed to oxyHb in vitro</td>
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<tr>
<td>Ohkuma, et al., 1997</td>
<td>dog double hemorrhage</td>
<td>measurement of arteriole dilation/constriction by using video microscopy</td>
<td>oxyHb attenuated propagation of arterioles by vasodilation</td>
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<tr>
<td>Ohkuma, et al., 1999</td>
<td>dog double hemorrhage</td>
<td>measurement of parenchymal arterioles w/ corrosion casts of pons</td>
<td>intraparenchymal portion of perforating arteries constricted after SAH</td>
</tr>
<tr>
<td>Ohkuma, et al., 2000</td>
<td>human aneurysmal SAH</td>
<td>measurements of intraparenchymal arterioles w/ corrosion casts of cortex</td>
<td>tapered narrowing &amp; decreased width of arterioles; decreased internal diameter &amp; increased wall thickness of arterioles</td>
</tr>
</tbody>
</table>

* CSF = cerebrospinal fluid; CT = computerized tomography; ICA = internal carotid artery; ICP = intracranial pressure; oxyHb = oxyhemoglobin; rCBF = regional CBF; rCBV = regional CBV; rCMRO₂ = regional cerebral metabolic rate of oxygen.
Even though a later study by Ohkuma, et al., examined the pontine region in a canine double-hemorrhage model, contradictory results were obtained, showing that extraparenchymal arteries were normal although intraparenchymal arterioles were constricted. A possible explanation may be that a lower pH in the blood clot in the extraparenchymal space might hyperpolarize membranes and reduce the contractile response of the small arteries or arterioles, even though more severe vasospasm occurs in the BAs, which are actually bathed in the same acidic blood clot.

Age can be another factor that influences vascular reactivity, especially in the microcirculation during cerebral vasospasm. Contractile responses of cerebral arteries to vasoconstrictive agents have been shown to be potentiated with age, which might underscore an increased incidence of symptomatic vasospasm in patients. Recently, Nakajima, et al., demonstrated in a rabbit model that vasospasm occurs in the BAs, in small extraparenchymal arteries, and in intraparenchymal arterioles of older animals. According to these authors, aging potentiated the degree of vasospasm in both BAs and in smaller arteries in the extraparenchymal space, and aging decreased the relaxant effect of papaverine. Interestingly, these authors observed vasospasm in the intraparenchymal arterioles in older rabbits but not in young ones. The presence of vasospasm in the small arteries of the brainstem and intraparenchymal arterioles in older rabbits coincides with symptomatic vasospasm and might predispose elderly animals to delayed cerebral ischemia.

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