Cerebral Pathophysiology in Hemorrhagic Shock. Nuclide Scan Data, Fluorescence Microscopy, and Anatomic Correlations

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SUMMARY Nineteen unanesthetized sheep were given Evans blue dye or radionuclide and then were incrementally bled into hemorrhagic shock (BP less than 50/25) by withdrawal of blood over a period of approximately one hour. Four sheep served as controls for histologic data. Static nuclide brain scans performed in 6 bled sheep demonstrated immediate uptake of the radionuclide. Fluorescence microscopic examination of brain tissue obtained at autopsy in bled sheep demonstrated dye uptake in the perivascular areas, but not in the neuropil. Electron microscopic examination of collateral cortical biopsy specimens showed swelling of perivascular astrocytic foot processes, but no endothelial abnormality. We feel that the uptake of radionuclide and dye-albumin complex by the perivascular astrocytes represents enhanced transendothelial transport rather than passage of molecules between endothelial cells. The significance of this presumably transient phenomenon is discussed.

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CEREBRAL DAMAGE and death caused by severe blood loss presents an important challenge for physicians. The tempo of blood loss is defined as a) acute (exsanguination), with blood loss and perhaps death occurring in minutes, b) subacute, with blood loss occurring over a period of one to several hours, and c) chronic, with blood loss occurring over a period of days or weeks.

Battlefield and roadway accidents causing exsanguination usually terminate fatally; patients with subacute blood loss may be viable, transportable, and treatable. We therefore studied the effects of subacute blood loss upon cerebral function in sheep. We are defining the pathophysiologic changes in that model, and hope to use the model for testing potential therapy.

Anoxic-ischemic disease produces variable effects upon the blood-brain barrier and upon cerebral histology. Ischemic cerebral damage tends to cause no immediate permeability of cerebral capillaries to albumin, unless there has been significant damage to the capillaries themselves, e.g., by oil or air emboli. Delayed permeability of the cerebral vasculature to albumin-sized particles in ischemic cerebral damage (stroke) is believed to be caused by ingrowth of new capillaries to aid in removal of necrotic debris, rather than by permeability of preexisting cerebral vessels. Therefore, we expect the nuclide brain scan performed soon after an episode of cerebral ischemia to be normal. This clinical study would not, however, detect permeability change that occurs during the creation of a cerebral lesion, or preclude its occurrence. Although the cerebral changes caused by anoxic-ischemic cerebral insults are well described, changes occurring during an insult caused by blood loss have been less often described or correlated with other data.

Materials and Methods

Animal Handling. Studies were performed on 23 female sheep, 10-13 months old, weighing 25-40 kg. Each sheep had been examined clinically by our veterinary surgeons, had a normal complete blood count, and was quarantined for 21 days. Each sheep was initially clipped and bathed, and was fasted for 48 hours (ruminant) prior to preparatory or experimental work.

Preparatory Work. Under Penthrane® anesthesia, bilateral femoral artery and vein catheters were inserted in 19 experimental sheep, advanced to the level of the diaphragm, filled with heparin solution (Lipo Hepin®), and clamped. In 4 of 7 sheep, non-occlusive catheters were placed in both common carotid arteries, filled with heparin solution, and clamped (see scan format). All catheters proved patent on the experimental day (see bone flap format). Postoperatively, each sheep was permitted to eat and drink ad libitum for at least 48 h, and then was offered only water for an additional 48 h prior to experimentation. No sheep appeared lethargic, ataxic, or ill.

Experimentation. On the experimental day, each of the 19 sheep was placed in a standing sling stall. The artery and vein catheters were unclamped, and the heparin in the catheters was withdrawn and replaced with heparinized saline solution (10 ml of Lipo Hepin/1,000 ml of normal saline). In 6 sheep, venous blood was serially withdrawn for measurements, including P02, pH, Pco2, pyruvate, lactate and NH3. In the 19 experimental sheep, polygraphic recordings of arterial blood pressure, pulse, and respiration were obtained. Electroencephalographic electrodes (standard clinical needle electrodes) were placed bilaterally in frontal and occipital areas, and runs of unipolar and bipolar activity were recorded every 5 min, and as indicated. (Machines used were Hewlett-Packard and Sanborn Biological Recording Monitors, and Beckman Offner Type T electroencephalograph.)
Twenty-five ml of 2½% Evans blue solution was given intravenously to each sheep. After observation lasting approximately 1 hr, each sheep was bled incrementally through the femoral venous catheters, 50 ml every 5 min, until clinical distress (as indicated by a blood pressure of approximately 50/25 plus associated signs of gasping and agitation) was noted. All sheep described in this study required withdrawal of 25–40 ml of blood/kg. Any sheep that tolerated significantly larger losses or developed distress earlier was considered unusual and excluded from the study. At the point of clinical distress, after appropriate measurements were taken (scan format and bone flap format), each sheep was rapidly sacrificed by injection of 5–10 ml of Lethol®. In each, the neck was rapidly incised, and the carotids were cannulated and injected with 200 ml of paraformaldehyde 4% in Millonig’s buffer. The brain was then rapidly removed.

Control Format. Four of the 23 sheep served as controls. In these, incremental bleeding was not performed. Each was rapidly sacrificed with Lethol and the brain removed after carotid perfusion had been performed.

Brain Scan Format. Seven sheep were studied by the scan format. In the 4 sheep in which bilateral carotid catheters were placed, 1.0 mc of Technetium 99 m pertechnetate-albumin complex* (prepared by our nuclear pharmacy) in 1.0 ml normal saline was injected into each carotid at the start of the experiment. Injections were repeated after 400–500 ml of blood had been withdrawn and again at the point of clinical distress. One min after each pair of injections, a 10,000 count scan was obtained.

The other 3 sheep received 10 mc of Technetium 99 m pertechnetate-albumin complex* intravenously, and then were positioned so that each sheep’s head was under the scanning crystal of the Pho Gamma III camera. Serial 10,000 count scans were obtained and examined for uptake by brain, salivary, and thyroid areas.

No sheep showed significant cerebral uptake of the radionuclide prior to bleeding. Salivary or thyroid uptake, considered to represent poor albumin binding of the technetium, was not noted. Each sheep had further brain scans performed during the incremental bleeding and at the point of clinical distress.

Bone Flap Format. Twelve sheep were studied by the bone flap format. On the preparatory day, after femoral artery and vein catheters were introduced, Penthrane anesthesia was continued in these sheep, and bilateral 3 × 3 cm craniectomies were performed over the posterior portions of the frontal bone, approximately 3 cm from the midline. A Hall air drill with slip clutch made the initial hole, and burr bits were used to complete the craniectomy. The dura was left intact, the wounds were lavaged with saline, and the skin was closed with Michelle’s clips. Postoperatively, no sheep appeared lethargic, ataxic, or ill.

On the experimental day, prior to incremental bleeding, under local anesthesia, the craniectomy wounds were opened and lavaged gently with normal saline. The dura was raised with a fine hook, incised with an iris scissor, and the open wounds were gently covered with saline-soaked gauze. All sheep tolerated this procedure without evident pain. The gauze was periodically remoistened. Immediately before incremental bleeding, a biopsy of the right cerebral cortex was performed using a plastic, suction-type biopsy tool. A piece of cerebral tissue measuring approximately 1.5 mm by 20 mm was obtained and immediately immersed in a Petri dish containing normal saline. The biopsy wounds bled for approximately 3 minutes. In no sheep used in this study was change in consciousness or diffuse EEG change noted. In no sheep was more than gentle packing required. Each sheep responded to the biopsy with a brief startle, and in no animal was continued distress or seizure activity produced. (No significant hematomas or subarachnoid hemorrhages were found at autopsy.) Incremental bleeding was then performed. At the point of clinical distress, a second cerebral biopsy was performed and placed in saline. Each sheep then was rapidly sacrificed.

Handling of Tissues. Tissues were examined grossly and prepared for light, electron, and fluorescence microscopy as follows: All necropsy specimens were serially sectioned parasagitally at 1–2 cm intervals and observed for lesions. Portions of frontal and occipital cortex, hippocampus, thalamic area, and cerebellum were mounted, stained with hematoxylin and eosin, cresyl violet or phosphotungstic acid–hematoxylin (PTAH) stain, and studied microscopically. The biopsy specimens were initially immersed in normal saline and divided with a razor blade into portions for light, electron, and fluorescence microscopy. The portions for light microscopy were stored in 4% paraformaldehyde, mounted, stained with H&E, cresyl violet, or PTAH, and studied. The portions for electron microscopy were diced and stored in 4% paraformaldehyde in Millonig’s buffer. The diced tissue was subsequently embedded in Epon, sectioned on a microtome, selected, thin-sectioned, and stained with lead citrate and uranyl acetate. All tissues were surveyed, and appropriate photographs were taken. The tissues from biopsy and necropsy specimens for fluorescence microscopy were frozen in liquid N₂ cooled isopentane, sectioned, and mounted. Using a Leitz light microscope, with appropriate condenser and exciter and barrier filters, tissues were examined for red fluorescence (Evans blue fluoresces red).*

Results

During subacute incremental bleeding blood pressure declined, but the pulse response was erratic (figs. 1, 2). The EEG tended to show slower rhythms as bleeding continued and blood pressure dropped (fig. 3). These findings have been described elsewhere. Radionuclide Scan Data. Nineteen sets of polaroid scintiphotographs were obtained in 7 sheep (table 1). Each was evaluated for cerebral uptake of Technetium 99 m pertechnetate complex. None of the 7 sheep had a defect in the blood-brain barrier during the control period. At the point of clinical distress, 3 sheep had...
definite (2+) uptake of radionuclide and, in 3 equivocal or minimal (1+) uptake was observed. The uptake was diffuse over the hemispheres. One sheep showed no uptake. Typical scans are shown (fig. 4).

Gross Cutting and Light Microscopy Data. Gross inspection and cutting of the 4 control specimens and the 7 scan format brains showed normal results. Twelve bone flap format brains showed slight subarachnoid blood over the right cerebrum (site of initial biopsy), and none over the left cerebrum. Serial cutting did not demonstrate intracerebral hematomas. The biopsy tracts extended through the cortex into the white matter but did not impinge upon thalamus or mid-brain. The tissue surrounding the right cerebral biopsy tracts (performed before onset of bleeding) tended to be blue-stained, whereas no definite blue staining was observed about the left (presacrifice) tracts. No other gross changes were seen. With the exception of tissues sampled from the biopsied areas, the light microscopic examination of biopsy and necropsy specimens demonstrated no pathological changes. No anoxic neurons were seen. Vacuolation was minimal or absent in all specimens. Most areas were free of red cells, confirming good washout by the perfusion-fixation technique. The biopsied areas showed the expected small areas of hemorrhage and tissue disruption.

Fluorescence Microscopy Data. Gross inspection of necropsy specimens showed blue staining of the meninges, but no areas of blue staining in the cerebral

**Figure 1.** Mean rate of blood loss (in cc/kg) in 19 sheep.

**Figure 2.** Effect of blood loss (in cc/kg) upon mean pulse rate (upper graph) and mean arterial blood pressure (lower graph). As is evident, correlation with blood pressure is almost linear, but there is no obvious relation with pulse.

**Figure 3.** Effect of blood loss (cc/kg) upon basic EEG rhythm. As blood loss continues, the normal low voltage fast activity (greater than 15 Hz) diminishes; theta (4 to less than 8 Hz) and then delta (1-4 Hz) rhythms increase.
Table 1: Results of 10,000 Count Nuclide Brain Scans in Seven Sheep Studied After Administration of Technetium-Albumin Complex by Either Intracarotid (IC) or Intravenous (IV) Route. Scoring was 0 = No Uptake (in Static Phase), 1 = Equivocal or Minimal, 2 = Definite, 3 = Marked.

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parenchyma were noted, except in the biopsied areas. These latter areas showed brilliant red, extravascular fluorescence when studied with the fluorescence microscope. The post-shock necropsy specimens showed no red fluorescence in the neuropil. The washed-out capillaries themselves, however, regularly appeared to fluoresce a faint red. The phenomenon could not be photographed. In contrast, the control necropsy specimens showed no red fluorescence in either neuropil or vessel walls. We therefore believe that this faint red fluorescence was of significance and not artifactual.

The biopsy specimens (pre- and post-shock) showed only a faint yellow fluorescence intravascularly, presumably due to autofluorescence of red blood cells. (Biopsied specimens did not have red cells washed out.)

Electron Microscopic Data. Electron microscopic examination was performed on 4 control necropsy specimens, 12 experimental prebleeding biopsy specimens, and 11 post-bleeding biopsy specimens. The results are summarized in table 2. Representative areas of gray and white matter were photographed with special attention to neurons and blood vessels. Inspection of all photomicrographs suggested that the post-bleeding biopsy specimens contained noticeably more neuronal cytoplasmic mitochondrial swelling and glial perivascular end foot swelling than did either of the other 2 groups. All 3 groups had variable amounts of nucleoprotein clumping and intra- and extracellular tissue holes, which were regarded as ar-

Figure 4. Static nuclide brain scans performed before, during and after blood loss to the point of clinical distress. Static vertex views, with snout at top of film, and ears at sides of film. Two left (prebleed) scans are normal; middle and two right scans show uptake of Tc-albumin complex diffusely in cerebrum. See also table 1.
The swollen glial end feet observed in the post-bleeding biopsy specimens did not contain vesicles. Intraglial mitochondria adjacent to the hydropic end feet appeared normal, in contrast to the neuronal mitochondria.

Several capillaries from all 3 groups were examined at magnifications of up to 100,000 times, and no abnormalities of the endothelium or the interendothelial junctions were noted. All capillary lumens appeared to be widely patent. There was no difference in the number of endothelial pinocytotic vesicles in the groups. Representative photomicrographs are shown (figs. 5-8).

**Discussion**

As described in the results, unanesthetized sheep incrementally bled to a point of clinical distress...

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**Figure 6.** Cortical biopsy, left cerebral hemisphere, sheep 338 (X 18,000) taken after bleeding detail of red cell, capillary and adjoining glial end foot, showing hydropic swelling of glial end foot.

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**Table 2. Evaluation of the Presence and Severity of Nucleoprotein Clumping (NNC), Neuronal Cytoplasmic Holes (NCH), Neuronal Mitochondrial Swelling (NMS), Neuropil Holes (NH), Glial Neuronal Clumping (GNC), Glial Cytoplasmic Holes (GCH) and Glial End Foot Swelling (GES). Features were scored 0 = Absent, 1 = Equivocal, 2 = Definite, 3 = Marked.**

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tifacts. All photomicrographs were formally interpreted for the presence of the following 7 features: 1) neuronal nucleoprotein clumping, 2) neuronal cytoplasmic holes, 3) neuronal mitochondrial swelling, 4) neuropil holes, 5) glial nucleoprotein clumping, 6) glial cytoplasmic holes, and 7) glial end foot swelling. Negative, equivocal, definite, and marked changes were scored as 0, 1+, 2+ or 3+ respectively. Inspection of the data suggests that artifactual changes were slightly more prevalent in the experimental biopsies than in the control necropsy specimens, and that definite neuronal mitochondrial swelling and glial end foot swelling were largely confined to the experimental post-bleeding biopsy specimens. The swollen mitochondria appeared to have increased matrix spaces, with minimal change of volume, membranes and crystal configuration.
Figure 5. Cortical biopsy, left cerebral hemisphere, sheep #394, taken after bleeding, detail of neuron (X 18,000), showing minimal artifactual change and early swelling of mitochondria.
demonstrated uptake of radionuclide on brain scanning. Evans blue fluorescence microscopy studies did not demonstrate gross albumin leak into the parenchyma (except in the areas in which biopsies were done), but showed slight uptake by the vessel-base ment membrane-glial cell complex. These awake sheep also showed electron microscopic evidence of mitochondrial matrix swelling and glial end foot swelling.

The pertechnetate ion, when injected intravenously, becomes 90% protein-bound, and unbound pertechnetate is taken up by the gastric mucosa, thyroid and choroid plexus, or excreted by the kidneys and other organs. Pertechnetate bound to albumin remains intravascular until the albumin molecule leaves the vascular compartment, or the pertechnetate diffuses off. Pertechnetate ion penetrates the normal brain capillary systems only slightly more rapidly than albumin does. Appreciable uptake of either pertechnetate or pertechnetate-albumin complex, therefore, implies an alteration in the blood-brain barrier. Quality control by our nuclear pharmacy was good, and the absence of detectable choroidal or thyroid uptake of radionuclide suggests that the phenomenon of increased cerebral uptake we described involved an albumin-sized rather than a crystalloid molecule.

The presence of albumin-sized molecules in the cerebral parenchyma, as demonstrated by a positive nuclide brain scan, is assumed to be due to passage of albumin and crystalloid between endothelial cells. Since the blood-brain barrier, as indicated by the nuclide brain scan, is negative immediately after diffuse ischemic infarction has taken place, an acutely positive static nuclide brain scan implies that the underlying cerebral lesion — trauma, embolus, hemorrhagic infarction, tumor, etc. — has directly damaged cerebral endothelium, either transiently (i.e., embolus, osmotic stress) or persistently. Delayed positivity of the brain scan, as in ischemic infarction, probably indicates growth into the damaged area of new capillaries without complete zonulae occludentes. These capillaries resemble systemic rather than cerebral capillaries in that they permit passage of albumin between endothelial cells.

Intravenously administered Evans blue solution is rapidly and firmly bound to serum albumin and is an experimental morphologic equivalent of the nuclide brain scan. Since Evans blue fluoresces red, fluorescence microscopy adds to visual inspection of serial brain slices in assessing brain vessel permeability to albumin. There is usually no detectable perivascular dye (albumin) leak immediately after or ischemic or anoxic brain injury. Occasionally, anoxic cerebral injury is followed by dye staining of the neurons, without pericapillary dye...
leaks or staining of the neuropil. This latter phenomenon may represent discrete transendothelial and transgial transport of dye-albumin complex rather than nonspecific leak of the dye-albumin complex between endothelial cells.

In our experiments, the acute cerebral uptake of radionuclide was not associated with loss of consciousness or with dye staining of the neurons or pericapillary parenchyma, (except in the areas where biopsies were taken) and was associated with faint fluorescence of well washed-out capillaries, and electronmicroscopic (EM) evidence of glial end foot swelling. This is compatible with localization of the nuclide in the glial end feet observed to be enlarged. A similar pattern of dye localization was noted by others after injection into the carotid artery of "barrier-damaging agents". Those workers concluded that the dye was localized to the area of the basement membrane. They also suggest discrete transport as an explanation for selective dye-staining of neurons (with dye-free neuropil) occasionally seen in experimental anoxic states. Transport of molecules through cerebral endothelium is an active process requiring a functioning rather than a damaged endothelium. We suggest that the increased transport of dye and nuclide we demonstrated is a response of intact and functioning endothelium to tissue hypoxia or hypovolemia, and probably exists only during the period of insult. Since patients are rarely administered radionuclides prior to their cerebral insults and then studied during the insults, this phenomenon of increased transport would not often be demonstrated clinically.

Transient, nonpersisting increases in cerebral parenchymal uptake of large molecules have been demonstrated experimentally after intracarotid injection of seran-polymer (in rabbits), after topical application of hypertonic solutions to pial vessels (in rabbits), after intracarotid injection of endotoxin (in rabbits), and during drug-induced seizures (in dogs). In the first (seran-polymer) model, the polymer was located in the neuropil, suggesting a leak between endothelial cells. It has been assumed that the intravenously administered albumin-bound dyes which stain the neuropil after topical application of hypertonic solutions do so by passing between endothelial cells. However, in dogs, these solutions have been shown to enhance transendothelial transport of sugars in the absence of dye leaks. It is tenable that enhanced transendothelial transport is occurring in the latter 3 models. Therefore, in cerebral lesions caused by metabolic rather than physical, i.e., embolic, agents, transient parenchymal dye or nuclide uptake may be caused by transcellular transport in addition to or instead of passage between endothelial
cells. In most clinical lesions, however, more persistent uptake of nuclide or contrast is largely or wholly due to passage of molecules between endothelial cells rather than transcellular transport.

Because cerebral capillaries demonstrate enhanced transport of albumin during clinical distress due to hemorrhagic shock, medications administered during that time, that are ordinarily excluded by the blood-brain barrier, may enter the neuropil; the effects of this may be beneficial or harmful. It is also possible that drug entry into the CNS may be enhanced by methods other than acute hemorrhagic shock. Drug entry into the central nervous system could be monitored by nuclide brain scanning.

The glial end foot swelling we described is explained by the role of glia in response to cerebral, presumably neuronal, injury, and specifically to cerebral anoxia. The lesion to which the glia are responding is tissue hypoxia, but some secondary mediator, such as catecholamine release or lactate accumulation, cannot be excluded. It is even possible that the glia are responding to a potential loss of intracranial vascular volume by the cerebral oligaemia, and that the increase in intracellular volume serves to keep constant the intracranial volume. This differs markedly from responses in other tissues, in which oligaemia causes mobilization of interstitial and intracellular fluids into the vascular space.

In hemorrhagic shock, the rate of blood loss may determine the nature and extent of cerebral injury. It has been demonstrated that experimental and clinical rapid exsanguination can cause focal, ischemic-appearing lesions, apparently at border zones between arterial territories, whereas subacutely evolving hemorrhagic shock causes a diffuse picture that is more compatible with anoxia than with hypoxia, but some secondary mediator, such as catecholamine release or lactate accumulation, cannot be excluded. It is even possible that the glia are responding to a potential loss of intracranial vascular volume by the cerebral oligaemia, and that the increase in intracellular volume serves to keep constant the intracranial volume. This differs markedly from responses in other tissues, in which oligaemia causes mobilization of interstitial and intracellular fluids into the vascular space.

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References

Low Molecular Weight Dextran
In Experimental Embolectomy

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SUMMARY The canine middle cerebral artery (MCA) was embolized with a pliable cylinder 8 mm long by 1.6 mm in diameter via the internal carotid artery. Both control and experimental embolectomies were performed 6 hours following embolization. The experimental animals were treated with low molecular weight dextran (LMD). In the control animals, the average volume of infarction in the brain was 1.45 cm$^3$. In the experimental animals the average volume of infarction was 0.13 cm$^3$.

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THE TREATMENT of cerebral thromboembolism is controversial. Anticoagulants, steroids, anti-edema agents, and hyperventilation are used in the management of cerebral ischemia, despite controversy over their benefits in clinical and experimental situations. Administration of barbiturates and dimethyl sulfoxide (DMSO) in experimental models has been encouraging. Operative treatment, such as middle cerebral artery (MCA) embolectomy, has been sporadically reported with variable success. It has been shown in experimental models that canine MCA embolectomy is safe within 5 hours of embolism, while embolectomy beyond 5 hours results in massive hemorrhagic brain infarction. As it is usually not feasible to perform embolectomy in humans within such a short period, several pharmacological agents have been employed experimentally to extend the "grace period."

Low molecular weight dextran (LMD) has been used in the management of acute stroke with conflicting results. In the present study, the effect of LMD was evaluated in dogs subjected to embolization followed by MCA embolectomy.

Method

Fourteen mongrel dogs weighing between 15 and 25 kg were divided into 2 groups: a control group of 9 animals, and an experimental group of 5.

Twenty-four hours before use, the embolus material was prepared from the following mixture:

- Microfil Orange Polymer,* Microfil 1.5 ml,
- Microfil Diluent 1 ml, Microfil Catalizer 1 ml, Silastic 382 Elastomer† 4.3 ml, Dow Corning Medical Fluid 360‡ 1 ml, Catalyst M† 0.1 ml, Tantalum powder 3 gm.

The embolus material was introduced into a glycerine-lubricated polyethylene 240 tube to make a cylinder 1.6 mm in diameter.

All animals in the study underwent the following procedures: the animals were anesthetized with intravenous sodium pentobarbital (25 mg/kg) and then intubated. Controlled respiration was maintained by a Harvard respirator. Arterial blood gases were checked frequently to maintain the pH, $P_{O_2}$, and $P_{CO_2}$ within physiological limits. An infusion of Ringer's lactate solution was started in the saphenous vein for fluid replacement. Blood pressure (BP), ECG, and EEG were recorded on a Grass Polygraph. A femoral artery catheter connected to a Statham transducer was used for BP measurement.

The bifurcation of the left common carotid artery was dissected and the internal carotid artery selectively catheterized with a 16-gauge intravenous cannula (Argile Medicut).‡ An embolus 8 mm long was inserted into the hub of a 10 ml syringe filled with

* Canton Biomedical Products, P.O. Box 2017, Boulder, CO 80302.
† Dow Corning Corp., Midland, MI 48640.
‡ Argile Medical, St. Louis, MO 63103.
Cerebral pathophysiology in hemorrhagic shock. Nuclide scan data, fluorescence microscopy, and anatomic correlations.

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