Brain Injury after Cerebral Arterial Air Embolism in the Rabbit as Determined by Triphenyltetrazolium Staining

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**Background:** Microscopic cerebral arterial air embolism (CAE) occurs commonly during cardiac surgery and causes acute and chronic nonfocal neurologic dysfunction. Nevertheless, most neuroimaging studies do not detect brain injury after cardiac surgery. Using a rabbit model, the authors hypothesized they could detect and quantitate severe brain injury and infarction 24 h after microscopic CAE using the vital stain triphenyltetrazolium chloride.

**Methods:** Experiments were conducted in methohexital anesthetized New Zealand white rabbits. Surgical shams (n = 5) underwent surgery but had no neurologic insult. Positive controls (n = 3) received 200 µl/kg of intracarotid air. Other animals were randomized to receive either 50 µl/kg of intracarotid air, which produces microscopic CAE (n = 18), or 300 µl intracarotid saline (control, n = 18). Outcomes included somatosensory evoked potential amplitude at 90 min, neurologic impairment score at 4 and 24 h (0 [normal] to 99 [comatose]), and percentage of nonstaining brain at 24 h using color-discrimination image analysis. Severely injured or infarcted brain does not stain with triphenyltetrazolium chloride.

**Results:** Surgical shams had little neurologic impairment and a small amount of nonstaining brain at 24 h (5.2 ± 2.4%; mean ± SD). Positive controls had profound neurologic impairment and large amounts of nonstaining brain (40–97%). Ninety-minute somatosensory evoked potential amplitude was less in animals receiving 50 µl/kg air versus saline: 38 ± 28% versus 102 ± 32%, respectively, P < 1 × 10−7. Neurologic impairment scores were greater in animals receiving 50 µl/kg air versus saline: at 4 h, 43 ± 16 versus 23 ± 9, P < 1 × 10−7; at 24 h, 24 ± 12 versus 15 ± 8, P = 0.013. Nevertheless, there was no difference between 50 µl/kg air and saline in nonstaining brain: 5.5 ± 2.9% versus 6.8 ± 5.4%, P = 0.83.

**Conclusions:** Neurologic injury after CAE is dose-dependent. Although microscopic CAE causes somatosensory evoked potential abnormalities and neurologic dysfunction, severe cerebral injury or infarction is not present at 24 h. The author’s findings are consistent with clinical imaging studies that suggest microscopic CAE causes neurologic dysfunction even though overt infarction is absent. (Key words: Image analysis; somatosensory evoked potentials.)

MICROSCOPIC cerebral arterial air embolism (CAE) occurs commonly during cardiac surgery and carotid endarterectomy. In each setting, microscopic CAE may contribute to perioperative neurologic injury. Pial window studies show injection of air into the rabbit internal carotid artery in volumes of 100 µl/kg or less results in microbubbles that stop flow in pial arterioles (50–200 µm diameter) for 1–6 min before restoration of blood flow. Using this as a model of microscopic CAE, we previously have shown that neurologic outcomes (evoked-potential recovery or neurologic impairment score7,8) are improved by prophylactic administration of doxycycline or heparin. We also have shown that, despite the presence of heparin, evoked-potential recovery after CAE is impaired during cardiopulmonary bypass as compared with the normal circulation. A limitation of our previous studies has been the absence of a direct measure of neuronal injury or necrosis. Therefore, we wished to develop a quantitative measure of brain injury after microscopic CAE.

To date, light microscopy studies after CAE have been descriptive only and vary widely in terms of the dose of air (10–1000 µl/kg), interval between CAE and examination (minutes, hours, or days), and severity of neuronal damage (none, moderate, or severe). These studies show that histologic abnormalities after CAE can be small (<1 mm), multifocal, widely distributed, and sometimes present contralaterally to the side of air injection. These features make light microscopy impractical as a method to quantitate.
neuronal injury after CAAE. Because lesion size and distribution are so highly variable, one would need to evaluate large numbers of fields (hundreds) in large numbers of sections (hundreds) to be sure all abnormalities were detected. As an alternative to light microscopy, we chose to investigate triphenyltetrazolium chloride (TTC) staining as a method to quantitate brain injury after CAAE. TTC has been used in numerous studies to delineate areas of severely injured brain. Regions of brain that do not reduce colorless TTC to its crimson reaction product (i.e., do not stain) correspond to areas of severe neuronal injury or infarction in animal models of focal cerebral ischemia\(^{15-17}\) and traumatic brain injury.\(^{18}\) Nonstaining (white/tan) brain is easily distinguished from crimson-stained brain because of the stark color difference.\(^{19}\) We wished to quantitate the fraction of brain tissue that did not stain with TTC 24 h after microscopic CAAE. We hypothesized that animals exhibiting signs of neurologic injury after microscopic CAAE (e.g., evoked-potential and neurologic abnormalities) would have a greater fraction of nonstaining brain than would controls.

**Materials and Methods**

Experimental protocols were approved by the Animal Care and Use Committee of the University of Iowa in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication 85-23, revised 1985).

**Group Assignments**

**Negative and Positive Controls.** We wished to establish outcome parameters (evoked-potential amplitudes, neurologic impairment scores, and TTC staining abnormalities) in animals that were true-negative (i.e., no neurologic insult and in true-positive (i.e., severe neurologic insult). Five New Zealand white rabbits served as a surgical sham group (true-negatives). Surgical shams underwent all procedures and evaluations as described herein, except that after carotid artery isolation the neck wound was closed without injection of either intracarotid air or saline. A different group of three rabbits received a large dose of intracarotid air, 200 µl/kg (true-positives). This dose of air was chosen on the basis of a previous study, wherein intracarotid injection of 150 µl/kg of air resulted in near-total loss of evoked-potential amplitude, severe early (3 h) neurologic impairment, and 100% 24-h mortality rate.\(^{20}\) We expected that brain injury would be so severe that unassisted survival would not be possible after 200 µl/kg intracarotid air. Thus, animals receiving 200 µl/kg intracarotid air underwent all procedures as described, except they remained intubated and ventilated and received continuous monitoring and intravenous fluids until spontaneous death, 16-20 h after air embolism.

**Study Groups.** Using computer-generated random numbers and a block design (n = 10 in each block), 40 New Zealand white rabbits (weight, 3.2-5.2 kg) were randomized to receive either intracarotid air (50 µl/kg; 9 females, 11 males) or intracarotid saline (300 µl; 11 females, 9 males). This smaller dose of air was chosen on the basis of a pial window study that found, in rabbits, intracarotid air in doses less than or equal to 100 µl/kg creates cerebral microbubbles (50-200 µm in diameter).\(^{6}\) A previous study from our laboratory indicated that 50 µl/kg intracarotid air results in mild to moderate neurologic impairment, but with a high rate of unsupported 24-h survival.\(^{20}\) These features are consistent with the clinical presentation of microscopic CAAE.

**Basic Animal Preparation**

Anesthesia was induced in nonfasting animals by inhalation of 5% isoflurane in oxygen. After cannulation of an ear vein with a 22-gauge catheter and orotracheal intubation with a 3.0-mm cuffled endotracheal tube, animals were briefly paralyzed with a single dose of intravenous succinylcholine (1 mg/kg). Thereafter, no muscle relaxants were administered. Animals were ventilated with 2% isoflurane in 30% oxygen and balance nitrogen to achieve normocapnia, monitored continuously with a calibrated anesthetic agent analyzer (Datex, Puritan-Bennett, Helsinki, Finland). Normal saline was infused intravenously at + ml · kg\(^{-1}\) · h\(^{-1}\). Rectal temperature was maintained at 38°C with a servocontrolled heating pad, but epidural temperature was monitored as an independent variable.

Animals were placed prone in a stereotaxic frame (Kopf Instruments, Tungunda, CA). The scalp was shaved. The skin was washed with povidone-iodine solution (Purdue Fredrick Co., Norwalk, CT) and all subsequent procedures were performed in a sterile fashion. After skin incision, a 2-mm Burr hole was drilled over the left frontoparietal cortex to expose dura. A 1-mm thermocouple (K-type, L0841902; Cole Parmer, Chicago, IL) was placed between the cranium and dura to monitor epidural temperature. The bone defect was filled with bone wax. Stainless steel screws for recording somatosensory evoked potentials (SSEP) were placed
into the skull with the active electrode located over the left parietal region, 6 mm lateral to the midline and 1 mm anterior to the coronal suture. The reference electrode was placed in midline into maxillary bone. Animals were turned supine and SSEP-stimulating needle electrodes were placed subcutaneously over the right median nerve. Through the left femoral artery, a saline-filled polyethylene catheter (PE-90; Intramedic, Parsippany, NJ) was advanced into the abdominal aorta for arterial pressure monitoring and intermittent blood sampling. After this, isoflurane was discontinued. Methohexital was administered as a bolus of 10 mg/kg intravenously, followed by a continuous intravenous infusion (1 mg/ml in saline) to give 15 mg · kg⁻¹ · h⁻¹. Methohexital was administered for the remainder of the experiment to avoid isoflurane inhibition of SSEP.

Cerebral Arterial Air Embolism

Through a midline neck incision, the left external, internal, and common carotid arteries were isolated, and a branch of the external carotid (usually the facial) was selected for cannulation. Other branches of the external carotid were ligated with 4-0 silk, and all bleeding points were cauterized. After a 15–20-min equilibration period, baseline (preembolism) physiologic measurements were obtained: epidural temperature, mean arterial pressure (MAP), arterial pH, partial pressure of carbon dioxide (PaCO₂), partial pressure of oxygen (PaO₂) (Il1304; Instrumentation Laboratory, Lexington, MA), hemoglobin concentration (Osm3), inhalation agents [rabbit absorption coefficients]; Radiometer, Copenhagen, Denmark], and plasma glucose concentration [Model 27; Yellow Springs Instrument, Yellow Springs, OH]. At this time, baseline SSEP measurements also were obtained. Thereafter (except in surgical shams), a temporary aneurysm clip was placed across the left common carotid just proximal to its bifurcation. A saline-filled PE-50 catheter (Intramedic; 39 cm long, 150 μl internal volume) was introduced retrograde through the facial branch of the external carotid artery into the proximal 1 to 2 mm of the internal carotid artery. Care was taken to avoid air entrapment in the facial or the internal carotid arteries. In animals randomized to receive intracarotid air, 50 μl/kg air was injected into the internal carotid, followed by a flush of 150 μl normal saline, both given at a constant rate of 3 μl/s by infusion pump. Thus, animals randomized to air received, in sequence, 0 μl saline (injection catheter dead space), 150 μl/kg air, and 150 μl saline flush into the internal carotid artery. In animals randomized to intracarotid saline 300 μl saline was injected into the internal carotid at 3 μl/s. Immediately after completion of the injection, the aneurysm clip was removed from the common carotid and the injection catheter was withdrawn into the external carotid, reestablishing continuity between the internal and common carotid arteries. The injection catheter was removed from the facial artery and the external carotid artery was ligated at its origin. SSEPs and physiologic data were recorded at 2, 5, 15, 30, 45, 60, 75, and 90 min after initiation of injection. In surgical shams (n = 5), the carotid system was not cannulated and SSEPs were recorded from the time of neck wound closure. In animals receiving a large dose of air (n = 3), 200 μl/kg air was given as described.

Somatosensory Evoked Potentials

SSEPs were derived by stimulation of the right median nerve. Using subcutaneous needle electrodes, supramaximal square-wave direct current pulses of 0.25-ms duration were delivered at 1.4 Hz. Sixty-four cortical responses were averaged with a Grass Model 10 evoked response system with band-pass filters of 0.3 and 10,000 Hz (Grass Instruments, Quincy, MA). High-amplitude electrical artifact automatically was rejected. The analog signal was converted to digital data by an A-D board interfaced with an IBM AT computer (Armonk, NY) for subsequent analysis. The amplitude of the primary cortical deflection was measured from the trough of the first major negative deflection (N₁, occurring at 13 ± 1 ms, n = 36) to the peak of the next positive deflection (P₁, occurring at 30 ± 4 ms, n = 36). N₁ and P₁ latencies were recorded for each animal before air or saline embolism; postembolism amplitude measurements were made from those points. The amplitude of the N₁-P₁ complex was expressed as a percentage of the baseline value for each animal.

Recovery and Neurologic Evaluation

Ninety minutes after air embolism, the arterial catheter, cranial screws, and cortical thermocouple were removed. Incisions were closed and infiltrated with a total of 3 ml lidocaine, 0.5%. The methohexital infusion then was discontinued. Animals were extubated when they regained spontaneous ventilation and protective airway reflexes. After extubation, animals briefly received 50% oxygen by mask. Neurologic status was assessed 4 h after air embolism. The neurologic scoring system used was a modification of that described by Baker et al. [21] (Table 1). The best possible neurologic score was 0; the worst possible neurologic score was 99. Using this scoring system, rabbits undergoing 10 min of global cerebral
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Table 1. Neurologic Scoring System

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Score</th>
<th>Maximum Abnormal Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of consciousness</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Clouded</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Stuporous</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Comatose</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Respiration</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Abnormal</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Cranial nerves</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Vision absent</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Light reflex absent (right)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Light reflex absent (left)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Corneal reflex absent (right)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Corneal reflex absent (left)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Facial sensation absent</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Auditory absent</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gag reflex absent</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Motor/sensory function</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Flexor response to pain in upper extremity absent (right)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Flexor response to pain in upper extremity absent (left)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Flexor response to pain in lower extremity absent (right)</td>
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<td></td>
</tr>
<tr>
<td>Flexor response to pain in lower extremity absent (left)</td>
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<td></td>
</tr>
<tr>
<td>Righting reflex absent</td>
<td>10</td>
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</tr>
<tr>
<td>Gait</td>
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<td>25</td>
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<tr>
<td>Normal</td>
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<tr>
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<tr>
<td>Moderate ataxia</td>
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<tr>
<td>Able to stand</td>
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<td></td>
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<tr>
<td>Unable to stand</td>
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<tr>
<td>No purposeful movement</td>
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<td></td>
</tr>
<tr>
<td>Behavior</td>
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<tr>
<td>Not grooming</td>
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<td></td>
</tr>
<tr>
<td>Not drinking</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Not exploring</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Worst possible score</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

This system was modified from Baker et al.\textsuperscript{21} Testing of the oculocephalic reflex was omitted, which, on the original scale, was worth 1 point.

ischemia during hypothermic conditions (29°C) were found to have less neurologic impairment, histopathologic change, and glutamate release than rabbits that were made ischemic while normothermic.\textsuperscript{21} Hence, this neurologic scoring system corresponds to histopathologic and biochemical indices of neurologic injury in this species. The neurologic examiner (J.C.) was aware of the protocol but was unaware of group assignment. Exubated animals were returned to the animal care unit, where food and water were available. Animals surviving to 24 h had a subsequent neurologic examination at that time. Animals receiving large-dose air embolism (200 μl/kg) were severely neurologically impaired despite discontinuation of methohexital. They were not returned to the animal care unit. They remained intubated and received continuous monitoring and intravenous fluid until spontaneous death. At death, brains were immediately harvested for TTC staining.

**TTC Staining**

After the 24-h neurologic evaluation, animals were reanesthetized with 5% isoflurane in oxygen and killed by pentobarbital overdose (150 mg/kg intravenously). Brains were quickly removed and chilled at −15°C for 15 min to aid sectioning. Brains were sliced into 2-mm coronal sections starting at the olfactory cortex. Cerebellum and medulla were not included because air was not expected to enter the circulation of these structures. The resulting sections (approximately 12 for each brain) were immediately immersed in 2% TTC (Fisher Scientific, Fair Lawn, NJ) in normal saline and incubated at 37°C for 30 min. Stained sections were protected from light and stored in 4% formalin until image analysis 1–3 days later. Sections were prepared for image acquisition and analysis as described subsequently.

**Image Analysis**

We required a method of image analysis that would differentiate and quantitate brain regions on the basis of color differences between viable (staining pink to crimson) and severely injured or infarcted (nonstained, white/tan) tissue with good spatial resolution. The method we (T.S.) developed uses an optical scanner with a resolution of 300 dpi (11.8 pixels/mm), yielding a nominal pixel size of 85 × 85 μm. (Thus, the smallest nonstaining area detectable by this system is 85 × 85 μm.) As described subsequently, we use National Institutes of Health (NIH) Image software (public domain, United States National Institutes of Health) to generate a table of colors present in the image. From this table, an operator identifies all colors judged to correspond to nonstaining tissue. Based on that selection, the computer then automatically identifies, highlights, and counts all pixels in the image of the same color, irrespective of spatial distribution. This allows for calculation of the percentage of nonstaining pixels in the image relative to the total number of pixels, giving the fractional area of nonstaining tissue.

Two-millimeter thick coronal sections of brain (or modeling clay, used for method validation) were sandwicched between two 15 × 15 cm nonglare transparent plastic sheets and arranged in a nonoverlapping fashion...
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(approximately 12 sections per sheet). Sheets were placed on the bed of an optical scanner, and dark green nonglossy paper was overlaid to serve as a background. Green was chosen as the background because there was no green coloration in any brain section. Each side of each sheet was scanned at a resolution of 300 dots/in and 24 bits of color using an Apple Color OneScanner (Apple Computer, Cupertino, CA) and Ofoto software version 2.0.2 (Light Source Computer Images, Lakeside, CA) on a Macintosh 6100 computer (Apple Computer). Thus, both sides of each section (anterior and posterior) were scanned, making the effective section thickness 1 mm. Images were stored on the computer hard drive as uncompressed Macintosh picture (PICT) files. Macintosh picture files were imported into Adobe Photoshop 4.0 (Adobe Systems, San Jose, CA). To correct for minor color errors created by scanning, color balance and brightness were adjusted so the image displayed on the monitor matched the actual colors of the sections. To be compatible with NIH Image 1.60 software the 24-bit color images were reduced (without dithering) to 8-bit color images using the adaptive palette generated by Adobe Photoshop 4.0.

Eight-bit color images were imported into NIH Image 1.60: scaling was set at 11.8 pixels/mm. When loaded into NIH Image, a look-up table (LUT) of 256 colors present in the image is automatically created and displayed by the software. Each of the 256 colors in the LUT comprises variable saturations of red, blue, and green; 0 represents the darkest possible value and 255 represents the lightest possible value for each of these three primary colors. Colors not in the image are not included in the LUT. We did not remove any colors from the LUT to ensure that every pixel was available for analysis. All green colors (background) in the LUT were first moved to the bottom of the LUT and were excluded from subsequent sorting procedures described herein. We considered pale colors (white to tan) to correspond to nonstaining, nonviable tissue. To aid in the identification of all pale pixels in the image, the remaining (nongreen) entries in the LUT were re-sorted according to total color saturation. To accomplish this, we summed the intensities of the red, blue, and green color saturations (redLUT, greenLUT, and blueLUT) of each individual nongreen color in the LUT using the macrolanguage present in NIH Image. These summed values constituted the total color density for each LUT entry. Then, these summed values were sorted on the basis of increasing value. A new LUT was created based on this sort (hereafter called the re-sorted LUT), which had a progression of colors from white through tan, to pink, to the darkest crimson, followed by a step change to greens (the background). This sorting process did not change the image to be analyzed, only the LUT subsequently used for analysis.

Using the density-slice tool of NIH Image it is possible to highlight all pixels in the image corresponding to any chosen individual LUT entry (i.e., color) or group of LUT entries. First, we measured the total number of pixels originating from the brain slices, both anterior and posterior surfaces. This was performed by selecting all non-green LUT entries from the re-sorted LUT. The total number of highlighted pixels in the image (total pixels originating from all sections) was recorded. Then the operator selected all colors in the resorted LUT that were considered to correspond to nonviable tissue (white through tan). The operator (J. C.) was unaware of group assignments and had passed a color-discrimination test (Munsell Color/GretagMacBeth, New Windsor, NY). In an iterative process, the operator optimized the range of selected pale LUT entries (colors) to avoid inclusion of brain regions that possessed any discernible pink coloration. Although less intensely stained than gray matter (crimson), normal white matter is stained a dark pink by TTC. Hence, normal white-matter regions were not automatically designated as nonstaining. The total number of highlighted pale (nonstaining) pixels was recorded. The percentage of pale pixels relative to the total number of pixels was calculated, giving the fractional area of injured tissue.

Model Brains

To assess the ability of this technique to measure fractional surface areas, we analyzed model brain sections made from known quantities of two colors of modeling clay, tan and pink. These colors were chosen to approximate the colors of nonstaining and staining brain, respectively. Each model brain weighed 10 g. Model brains with four different proportions of tan and pink (by weight) were made: 1% tan/99% pink; 10% tan/90% pink; 90% tan/10% pink; and 99% tan/1% pink. The clay was broken into small pieces and randomly mixed to form a shape approximately that of a rabbit brain. Model brains were coronally sectioned in 2-mm thick slices (fig. 1). Sections underwent image analysis as described previously. After each image acquisition, each model brain again was broken into small pieces and remixed, resulting in a new spatial distribution of colors. The clay then was remodeled, sectioned, and imaged again for a total of five image sets for each model brain.
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Fig. 1. Representative sections of model clay brains containing, from top to bottom, 1, 10, 90, and 99% tan clay.

The measured percentage of tan pixels was compared with the known percentage of tan clay (by weight) in the model brains.

Statistical Analysis

Data are reported as the mean ± SD. The correlation between known and measured percentages of tan clay in model brains was assessed using the Pearson correlation coefficient. Outcome endpoints were SSEP recovery at 90 min, 4 h, and 24 h; neurologic impairment scores; and percentage of nonstaining brain. We tested whether outcomes differed between the air and saline groups using the two-sided Wilcoxon–Mann–Whitney test (StatXact 3 for Windows; Cytel Software Corporation, Cambridge, MA). As a supplemental secondary analysis, analysis of covariance was used to screen for any relation between systemic physiologic variables and magnitude of final SSEP recovery (SYSTAT 6.0 for Windows; SPSS Incorporated, Chicago, IL).

Results

Image Analysis Validation

As shown in figure 2, there was an excellent correlation between the measured percentage of tan pixels and the actual percentage of tan clay in model brains: \( R^2 = 0.999 \), \( \text{slope} = 0.99 \), \( \text{Y intercept} = -0.19\% \) (not significantly different than 0). Bland–Altman analysis (fig. 2) shows that absolute errors in measured tan may vary with the actual percentage of tan but are so small as to be negligible. Thus, the image-analysis system is able to accurately measure fractional surface areas of regions with irregular shapes and distributions based on color differences.

Negative and Positive Controls

The five surgical shams (true-negatives) had a 90-min SSEP amplitude of 112 ± 29% (median = 100%) of baseline, a 4-h neurologic impairment score of 15 ± 5% (median = 14), a 24-h neurologic impairment score of 15 ± 5 (median = 19), and 24-h nonstaining TTC area of 5.2 ± 2.4% (median = 6.2%). The three rabbits that received large-volume CAEE (200 µg/kg; true-positives) had 90-min SSEP recoveries of 0, 5, and 0%; 4-h neurologic impairment scores of 96, 88, and 96; and nonstaining TTC areas (16–20 h after CAEE) of 61.3, 40.0, and 96.8%. Representative coronal sections of TTC-stained brains and an example of the display and re-sorted LUT created by NIH Image are shown in figures 3A and B.

Fifty Microliters/kilogram Air versus Saline

Four animals were excluded before analysis. Two of 20 animals randomized to 50 µl/kg intracarotid air exhibit-
ported marked hypertension immediately after air injection, followed by hypotension for the rest of the experiment. No other animals in this group had this response. Acute hypertensive responses to intracarotid air are caused by air entry into the brain stem arterial circulation.22 Because brain stem ischemia might adversely affect 2-hr survival, these animals were excluded. Two animals in the saline group were excluded because of surgical bleeding in one and a postoperative airway accident in another.

Systemic physiologic variables for animals receiving intracarotid air and saline are shown in table 2. There were no important differences between groups in any variable. The effect of air and saline injection on SEP is shown in figure 4. In all animals receiving 50 μL/kg intracarotid air, SEP was totally abolished at 2 and 5 min after the start of injection, with a gradual partial recovery over time. After saline injection, the SSEP signal was never abolished. At 90 min after embolization, SSEP amplitude recovered to 38 ± 28% (median = 35%) of baseline in animals receiving air (n = 18) versus 102 ± 32% (median = 99%) in animals receiving saline (n = 18), P < 1 × 10⁻⁷. In both groups, 90-min SEP recovery did not significantly depend on the absolute amplitude of baseline SEP (μV); gender, or epidural temperature, arterial pressure, arterial oxygen pressure (PaO₂), hemoglobin concentration, or plasma glucose averaged over the 90-min recording period. At 4 h after embolization, animals receiving air had greater neurologic impairment scores (43 ± 16, median = 41, n = 18) than animals receiving saline (25 ± 9, median = 20, n = 18), P < 1 × 10⁻⁷. Four animals died before 24-h neurologic examination: 3 of 18 animals randomized to air (4-h neurologic impairment scores of 42, 61, 97), and 1 of 18 animals randomized to saline (4-h neurologic impairment score of 14). Because post-procedural deaths are not necessarily on a neurologic basis, 24-h neurologic scores were assigned.
only to surviving animals. At 24 h after embolization, animals receiving air had greater neurologic impairment scores (24 ± 12, median = 25, n = 15) than animals receiving saline (15 ± 8, median ± 14, n = 17); P = 0.013. Despite differences in SSEP and neurologic impairment, there was no difference between air (5.5 ± 2.9%, median = 4.6%, n = 15) and saline (6.8 ± 5.4%, median = 3.5%, n = 17) groups in the percentage of nonstaining tissue at 24 h (P = 0.83). Representative coronal sections of TTC-stained brains from each group and an example of the display and re-sorted LUT created by NIH Image are shown in figures 3C and D.

Discussion

Dose-dependent Outcomes after Clinical CAAE

The clinical sequelae of CAAE are dose-dependent. When large volumes of air enter the cerebral circulation, seizures, hemiparesis, cerebral infarction, coma, and often death result.23-28 Fortunately, large-volume CAAE is a rare clinical occurrence. In contrast, microscopic CAAE is now recognized as commonplace in patients undergoing cardiac surgery,1,2 carotid endarterectomy,3 or cerebral angiography29 and in patients with artificial heart valves.30 Although often subtle in presentation, micro-

Table 2. Systemic Physiological Variables: 50 µL/kg Air versus 300 µL Saline

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Baseline (Time after Embolus (min)) 30</th>
<th>Baseline (Time after Embolus (min)) 60</th>
<th>Baseline (Time after Embolus (min)) 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidural temperature (°C)</td>
<td>Air</td>
<td>38.2 ± 0.5</td>
<td>38.4 ± 0.5</td>
<td>38.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>38.1 ± 0.2</td>
<td>38.3 ± 0.2</td>
<td>38.3 ± 0.2</td>
</tr>
<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>Air</td>
<td>91 ± 10</td>
<td>95 ± 10</td>
<td>91 ± 10</td>
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<tr>
<td></td>
<td>Saline</td>
<td>90 ± 9</td>
<td>95 ± 9</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>pH_a</td>
<td>Air</td>
<td>7.41 ± 0.02</td>
<td>7.40 ± 0.03</td>
<td>7.40 ± 0.02</td>
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<tr>
<td></td>
<td>Saline</td>
<td>7.42 ± 0.02</td>
<td>7.41 ± 0.03</td>
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<tr>
<td>PaCO_2 (mmHg)</td>
<td>Air</td>
<td>40 ± 2</td>
<td>39 ± 2</td>
<td>39 ± 2</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>39 ± 2</td>
<td>39 ± 2</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>PaO_2 (mmHg)</td>
<td>Air</td>
<td>167 ± 16</td>
<td>167 ± 15</td>
<td>160 ± 14</td>
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<td>Saline</td>
<td>173 ± 18</td>
<td>169 ± 16</td>
<td>169 ± 19</td>
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<tr>
<td>Hemoglobin (g/dl)</td>
<td>Air</td>
<td>12.5 ± 0.8</td>
<td>12.3 ± 1.0</td>
<td>12.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>12.3 ± 1.2</td>
<td>12.1 ± 1.1</td>
<td>11.9 ± 1.1</td>
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<tr>
<td>Glucose (mM)</td>
<td>Air</td>
<td>7.9 ± 2.7</td>
<td>6.5 ± 2.2</td>
<td>5.8 ± 1.9</td>
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<td>Saline</td>
<td>7.5 ± 2.7</td>
<td>6.4 ± 2.1</td>
<td>6.3 ± 1.8</td>
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Values are mean ± SD; n = 18 in both groups.

Fig. 4. Somatosensory evoked potential amplitudes over time after intracarotid embolism of either 300 µL saline (left) or 50 µL/kg air (right); n = 18 in each group. Missing data points are caused by overlap.

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scopic CAAE nevertheless can result in neurologic impairment. At 24 h after cardiac surgery, neurologic signs from CAAE derived from oxygenator microbubbles (which range from 50 μm to 200 μm in diameter) are nonfocal and consist of drowsiness, incoordination, nystagmus, and depressed reflexes. Although these signs largely resolve over several days, neuropsychologic testing reveals persistent abnormalities that are proportional to the number of microscopic CAAEs occurring during surgery.\textsuperscript{1}

**Dose-dependent Outcomes after Experimental CAAE**

In the rabbit model of CAAE, neurologic outcomes after CAAE are also dose-dependent. In this and a previous\textsuperscript{30} study, we found larger volumes of intracarotid air (150–200 μl/kg) to result in poor SSEP recovery, severe neurologic impairment, and a high 24-h mortality rate. In the current experiment, injection of 200 μl/kg air caused severe and extensive cerebral injury, as indicated by irreversible loss of SSEP amplitude, profound neurologic impairment, and a large percentage of brain that failed to stain with TTC. Therefore, in this species, administration of 150 μl/kg or more of intracarotid air results in a picture that closely approximates the clinical effects of large-volume CAAE.

In contrast, 50 μl/kg intracarotid air, which creates cerebral microbubbles in this model,\textsuperscript{6} is associated with moderate SSEP recovery, lesser degrees of neurologic impairment, and a much greater rate of 24-h survival. At 24 h after small-volume CAAE (50 μl/kg), neurologic abnormalities are global in character (reduced level of consciousness and spontaneous activity) and seizures are not observed. These signs are consistent with the clinical presentation of microscopic CAAE. Compared with saline controls, animals receiving 50 μl/kg air had significantly greater SSEP abnormalities and greater neurologic impairment scores at 4 and 24 h. Nevertheless, image analysis of TTC-stained sections did not reveal extensive brain injury in animals receiving 50 μl/kg air. In fact, there was no difference in the percentage of nonstaining (presumably injured) tissue between animals receiving air and the saline controls. Post hoc power analysis indicates that we used a sufficient number of animals to have had a 95% chance of detecting a 2.5% increase in nonstaining tissue.

Using model brains, our image analysis technique resulted in excellent agreement between actual and measured percentages of tan clay. Therefore, it seems unlikely that the lack of difference between air and saline groups in the percentage of nonstaining tissue was caused by inadequate precision in the image analysis method. Admittedly, however, the optical resolution of this system was limited to one pixel (85 × 85 μm). If microscopic CAAEs result in areas of severe injury or infarction smaller than this, then our image analysis method would not be capable of detecting them. Nevertheless, even if not individually identifiable, widely distributed nonstaining areas might produce regions that would appear to be only lightly stained with TTC, i.e., light pink rather than crimson. Therefore, in blinded fashion and in duplicate, we compared the percentages of nonstaining or lightly staining (light pink) tissue between air and saline groups using a manual tracing technique in NIH Image. We again observed no difference between animals receiving 50 μl/kg air (3.9 ± 4.5%, median = 3.1%) and those receiving saline (6.7 ± 7.7%, median = 5.0%). We conclude, therefore, that although microscopic CAAEs (50 μl/kg intracarotid air) result in neurologic impairment at 24 h, neuronal injury sufficient to result in even small differences in TTC staining does not occur.

**Early versus Late Outcomes after Microscopic CAAE**

Because neuronal necrosis or apoptosis sometimes can require days to weeks to fully mature, it is entirely possible that survival periods greater than 24 h may be needed to detect severe injury or infarction after microscopic CAAE. Conversely, neurologic impairment after microscopic CAAE is usually greatest earlier after the insult (24 h), not later.\textsuperscript{1} Because widespread cerebral TTC-staining abnormalities were reported 6 h after a large dose of intracarotid air in pigs,\textsuperscript{51} and because the clinical course after microscopic CAAE is generally one of steady improvement (not late deterioration), we hypothesized that TTC-staining abnormalities would be more likely to be present 24 h after microscopic CAAE than days later. Contrary to our hypothesis, however, there was no sign that brain injury after 50 μl/kg intracarotid air (microscopic CAAE) was any worse than after intracarotid saline. This observation is consistent with clinical studies. With only one exception,\textsuperscript{52} magnetic resonance imaging studies conducted 1–4 weeks after cardiac surgery do not reveal new widespread focal abnormalities, watershed infarcts,\textsuperscript{33–47} or new discrete changes that correspond with either encephalopathic symptoms\textsuperscript{34} or microscopic CAAE.\textsuperscript{35} If microscopic CAAEs (or other insults) occurring during cardiac surgery were to result in a substantial amount of delayed cerebral necrosis, such regions should have been readily detected in the clinical imaging studies. Thus, micro-
scopnic CAAEs either result in brain lesions so small as to also be undetectable by magnetic resonance imaging, or perhaps, as our results suggest, largely result in functional abnormalities (electrophysiologic, neurologic status) rather than infarction. In either case, our results suggest that, currently, there may not be a clinical imaging feature that would readily identify or correspond to neurologic dysfunction produced by microscopic CAAE.

Small-dose versus Large-dose CAAE

Because of the dramatic difference in the severity of neurologic injury that occurred with the two doses of air used in this study (50 and 200 μL/kg), we propose that the pathophysiology of CAAE may critically depend on the dose of air or the size of the resulting bubbles. Microbubbles (50-200 μm in diameter) are rapidly absorbed (after 1-11 min) and, as a result, interrupt cerebral arteriolar flow only briefly. When we administer 50 μL/kg air into the rabbit carotid, the SSEP signal initially is obliterated, indicating that cerebral blood flow is near zero. However, SSEP recovery starts within 10-15 min after air injection and is near maximal by 30 min. Bubbles compatible with these recovery times have radii on the order of 100 μm. Our previous studies and work from other groups suggest neurologic injury from microscopic CAAE probably is not the result of the brief ischemic period but instead results, at least in part, from the thrombotic or inflammatory processes that occur with reperfusion. In contrast, when larger bubbles are created, substantially more time is needed for their absorption. For example, bubbles with diameters of 500 and 1000 μm require 85 and 250 min to be absorbed, respectively. When we give the larger volume of air (200 μL/kg), there is no SSEP recovery. This suggests either that there is no restoration of cerebral blood flow after air injection whatsoever or that by the time cerebral blood flow is (at least partially) restored, irreversible neuronal injury has occurred. In either event, 200 μL/kg air clearly results in relatively long ischemic times, which is consistent with blockade of larger conducting arteries. Hence, in the setting of a large-volume CAAE, larger bubbles probably stop flow in larger conducting arteries for periods sufficiently long to cause primary ischemic injury.

Biochemical Basis of TTC-staining Abnormalities

The conversion of colorless TTC to its crimson reaction product depends on the presence of reduced nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). NADH or NADPH donates electrons to TTC, chemically reducing it to a colored formazan. The presence of NADH or NADPH, in turn, depends on the functionality of linked mitochondrial or cytosolic dehydrogenases. When mitochondria are so damaged that membrane-bound dehydrogenases are dysfunctional, mitochondrial respiration ceases and the processes of cellular energy failure and necrosis ensue. It is for this reason that regions failing to stain with TTC (i.e., lacking dehydrogenase activity and NADH) correspond to regions of infarction. However, several light microscopy studies have described brain regions that were clearly abnormal with hematoxylin and eosin staining (albeit not infarcted) that could continue to convert TTC to its colored product. Thus, during some conditions, neuronal damage and dysfunction may occur, but mitochondrial activity recovers or remains largely intact. Because rabbits receiving 50 μL/kg air had unequivocal electrophysiologic and neurologic abnormalities compared with saline controls, we conclude that microscopic CAAE can result in neurologic injury or dysfunction but without either overt infarction or gross disruption of cellular respiratory activity.

Surgical shams, animals that underwent anesthesia and surgery without air or saline embolism, had a low (but not zero) percentage of pixels identified as nonstaining at 24 h. There were no recognized procedural difficulties that might explain focal neurologic injury in these animals. In a study comparing light microscopy with TTC staining, Park et al. also noted that surgical shams often had small patches of nonstaining tissue that appeared histologically normal. We cannot readily explain the presence of such areas. We also note that animals that received saline injection in the internal carotid had variable SSEP responses and, in some animals, indications of neuronal injury by TTC staining. Although there was never a total loss of SSEP signal after saline embolism, some animals had sustained reductions of SSEP amplitude. We speculate that temporary unilateral carotid occlusion and infusion of a non-oxygen-carrying solution (saline) may, in some animals, create regions that are briefly ischemic or anoxic, and this may cause some neuronal injury detectable by TTC at 24 h.

Summary

In summary, neurologic outcomes from CAAE are dose-dependent. Large volumes of air result in severe neurologic impairment and widespread TTC-staining ab-
normalities. In contrast, small-volume (microscopic) CAAEs result in SSEP abnormalities and neurologic dysfunction but, at 24 h, brain injury is not sufficiently severe to result in cellular respiratory failure or infarction as detected by TTC staining. Our results suggest that clinical imaging studies may not detect structural abnormalities after microscopic CAAE.

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