J Neurosurg 106:687–694, 2007

Protection of mitochondrial function and improvement in cognitive recovery in rats treated with hyperbaric oxygen following lateral fluid-percussion injury

Zhengwen Zhou, M.D.,[1,2] Wilson P. Daugherty, M.D., Ph.D.,[1] Dong Sun, M.D., Ph.D.,[1] Joseph E. Levasseur, M.S.,[1] Nabil Altememi, B.S.,[1] Robert J. Hamm, Ph.D.,[3] Gaylan L. Rockswold, M.D., Ph.D.,[4] and M. Ross Bullock, M.D., Ph.D.[1]

Departments of [1]Neurosurgery and [3]Psychology, Virginia Commonwealth University School of Medicine, Richmond, Virginia; [4]Department of Neurosurgery, Hennepin County Medical Center, Minneapolis, Minnesota; and [2]Department of Neurosurgery, RenJi Hospital, Shanghai Second Medical University, Shanghai, China

Object. Hyperbaric oxygen (HBO_2) has been shown to improve outcome after severe traumatic brain injury, but its underlying mechanisms are unknown. Following lateral fluid-percussion injury (FPI), the authors tested the effects of HBO_2 treatment as well as enhanced normobaric oxygenation on mitochondrial function, as measured by both cognitive recovery and cellular adenosine triphosphate (ATP) levels.

Methods. Adult male Sprague–Dawley rats were subjected to moderate lateral FPI or sham injury and were allocated to one of four treatment groups: 1) FPI treated with 4 hours of normobaric 30% O_2; 2) FPI treated with 4 hours of normobaric 100% O_2; 3) FPI treated with 1 hour of HBO_2 plus 3 hours of normobaric 100% O_2; and 4) sham-injured treated with normobaric 30% O_2. Cognitive outcome was assessed using the Morris water maze (MWM) on Days 11 to 15 after injury. Animals were then killed 21 days postinjury to assess hippocampal neuronal loss. Adenosine triphosphate was extracted from the neocortex and measured using high-performance liquid chromatography. The results showed that injured animals treated with HBO_2 or normobaric 100% O_2 alone had significantly higher levels of cerebral ATP as compared with animals treated using normobaric 30% O_2 ($p \leq 0.05$). The injured animals treated with HBO_2 had significant improvements in cognitive recovery, as characterized by a shorter latency in MWM performance ($p \leq 0.05$), and decreased neuronal loss in the CA2/3 and hilar regions as compared with those treated with 30% or 100% O_2 ($p \leq 0.05$).

Conclusions. Both hyperbaric and normobaric hyperoxia increased cerebral ATP levels after lateral FPI. In addition, HBO_2 treatment improved cognitive recovery and reduced hippocampal neuronal cell loss after brain injury in the rat.

Key Words • traumatic brain injury • fraction of inspired oxygen • hyperbaric oxygen treatment • cognitive function • adenosine triphosphate • hippocampal cell count • rat

Traumatic brain injury consists of primary and secondary insults that result in cell death and dysfunction. The primary insult is due to transient mechanical tissue damage, whereas the secondary insults consist of delayed interrelated biochemical events that lead to ischemia, hypoxia, elevated ICP, reduced cerebral blood flow, excitotoxicity, and metabolic failure.[32] Data from both clinical and experimental studies have implicated the hippocampus as an important structure for memory.[12,50] This structure has been found to be particularly vulnerable to many types of secondary insults, such as ischemia, hypoxia, hypoglycemia, and experimental brain injury.[3,12,18,27,44,47] Among these injuries, cerebral hypoxia and ischemia are believed to contribute significantly to the secondary insult process even after the initial traumatic insult has ceased.

Hypoxia and ischemia cause a shift away from aerobic metabolism and limit much of the aerobic ATP production, with a shift to glycolysis, resulting in an enormous rise in cerebral lactate levels, which in turn lowers brain pH. An imbalance between energy demand and availability results in the consequent ischemia-like state, with a reduction in the ATP available to neurons and glia.[21] Given that hypoxia and ischemia are major pathophysiological complications after TBI, it is not surprising that 80 to 90% of patients who die of head injury demonstrate ischemic changes on histopathological examination of brain tissue.[18] It has been demonstrated in human studies that ischemia partly

Abbreviations used in this paper: ADP = adenosine diphosphate; AMP = adenosine monophosphate; ANOVA = analysis of variance; ata = atm absolute; ATP = adenosine triphosphate; DHR = dihydrorhodamine 123; FPI = fluid-percussion injury; HBO_2 = hyperbaric oxygen; HPLC = high-performance liquid chromatography; ICP = intracranial pressure; MWM = Morris water maze; NAD = nicotinamide adenine dinucleotide; NADH = reduced form of nicotinamide adenine dinucleotide; ROS = reactive oxygen species; SEM = standard error of the mean; TBI = traumatic brain injury.

results from a severe reduction in cerebral blood flow early after the primary injury.[6] Furthermore, arterial hypoxia doubles the mortality rate in these hypoxic patients.[9] For this reason, the manipulation of brain O_2 delivery may be a means of treating cerebral hypoxia.

There are several ways to enhance O_2 delivery, including increasing cerebral perfusion pressure with amines as well as increasing relative blood flow.[2] Methods that raise cerebral perfusion pressure may have inherent complications, such as increasing the possibility of intracranial hemorrhage or causing adult respiratory distress syndrome and acute renal failure.[41] In the current study we used methodologies that enhance O_2 solubility in plasma by either increasing the PaO_2 using HBO_2 or increasing the O_2 tension by simply increasing the inspired fraction of O_2 to 100% (normobaric hyperoxia).

In the past four decades, HBO_2 has been used in the treatment of cerebral lesions, such as those caused by experimental ischemia[22,40] and human head injury.[41,42] Although the potential mechanism of HBO_2 in treating TBI is not fully understood, the use of O_2 enhancement in TBI is based on the theory that mitochondrial injury impairs the production of energy substrates such as ATP and that, at least in some cells, this reaction may be reversible. Improving O_2 availability may thus stimulate the cells to recover function by reactivating them metabolically, thereby allowing restoration of ion pumping, resting membrane potential, electrical activity, and normalization of astrocyte volume.

We have previously demonstrated that HBO_2 treatment can increase injured brain tissue PO_2 immediately after injury, enhance the mitochondrial redox potential at 4 hours postinjury, and increase brain tissue O_2 consumption.[13] To further explore the therapeutic potential of HBO_2, we hypothesized that if it were able to improve cerebral aerobic metabolism after TBI, it would do so by increasing cerebral ATP levels. High-performance liquid chromatography was used to measure ATP levels in cerebral tissue. Furthermore, we evaluated the effect of HBO_2 treatment on cognitive recovery by using the MWM test and assessed hippocampal neuronal cell loss by using an unbiased stereological method. We also measured free radical production following hyperoxia treatment. We have thus used several of the most highly specific assessment methods available for TBI studies in animals to validate the anatomical and mechanistic bases of acute oxygenation therapies in diffuse TBI.

Materials and Methods

Animal Population

Adult male Sprague–Dawley rats (Harlan, Inc.) weighing 290 to 350 g were used in this study. Rats were housed singly in an environmentally controlled room in the animal facility, with free access to food and water, and were maintained on a 12/12-hour light/dark cycle. All experiments were approved by the Institution of Animal Care and Use Committee of Virginia Commonwealth University in accordance with all guidelines for animal use in research. Ninety-two animals were used and divided into four experimental groups: 1) sham-injured animals receiving normobaric 30% O_2, 22 rats; 2) injured animals receiving normobaric 30% O_2, 23 rats; 3) injured animals receiving normobaric 100% O_2, 24 rats; and 4) injured animals receiving HBO_2, 23 rats. These animals were allocated to three experiments: 1) ATP production, four rats from each experimental group; 2) cognition, 10 rats from each experimental group; and 3) free radical generation: eight from the sham-injured group, 10 from the 100% O_2 group, nine from the HBO_2 group, and nine from the 30% O_2 group.

Induction of Anesthesia

Animals received a gas mixture of O_2 and N_2 via mechanical ventilation. We used 30% O_2 and 70% N_2 as nonexperimental gases because this mixture is close to that used in an intensive care unit in which most patients with TBI are treated. Moreover, 30% O_2 is relatively close to the room air equivalent of 21%. Isoflurane anesthesia was provided via the carrier gas mixture and adjusted according to the protocol described later in this paper.

Surgical Procedures

The lateral FPI model has been previously described in detail.[14,31,33] Briefly, rats were anesthetized using 4% isoflurane in a carrier gas mixture of 70% N_2 and 30% O_2. The animals were immediately intubated, received mechanical ventilation with isoflurane reduced to 2%, and then were secured in a stereotactic frame (Kopf Instruments). The animal's core temperature was maintained at 37.0 ± 0.5°C by using an animal homeothermic heating system. A midline scalp incision was made, and the soft tissue was reflected to reveal the bregma, lambdoid, and sagittal suture lines as well as the parietal ridges. A 4.8-mm craniotomy was trephined into the left parietal bone, with the anterior edge of the craniotomy being located at the bregma −4.0 mm and centered between the sagittal suture and the parietal ridge. Two 1/16th-in stainless steel screws were anchored in bur holes 1 mm rostral to the bregma and 1 mm caudal to the lambdoid suture lines. A modified Luer-Loc needle hub (machine cut to an 8-mm length and a 2.6-mm inside diameter) was placed over the intact dura mater and bonded to the skull with α-cyanoacrylate glue. The hub and screws were then stabilized with dental acrylic.

After the dental acrylic sufficiently hardened, the animal was briefly removed from anesthesia to administer a 2.25 ± 0.04–atm fluid-percussion pulse and then reconnected to the anesthesia. This injury level was used to produce a moderately severe injury that is generally associated with prolonged cognitive deficits. After the injury, the cranial connector assembly was removed from the skull, and the scalp wound was sutured closed. Sham-injured animals underwent identical anesthesia and surgeries but did not actually receive the FPI.

Hyperoxia Treatment: HBO_2 and 100% O_2 Without HBO_2

Experimental hyperoxia was induced using 4 hours of normobaric 100% O_2 alone or 1 hour of HBO_2 followed by 3 hours of normobaric 100% O_2. In the HBO_2 treatment group, following the injury, animals received ventilation at 30% O_2 for 15 minutes before being transferred to a hyperbaric research chamber (model B11-22, Reimers Engineering, Inc.) pressurized to 1.5 ata with 100% O_2. Animals were then allowed to remain in the chamber breathing 100% O_2 at less than 1 ata for another 3 hours. The O_2 concentration in the chamber was monitored with an O_2 meter (Mini OX 3000, MSA Medical Products) to ensure 100% O_2 throughout the treatment. In the normobaric 100% O_2 treatment group (without HBO_2), following the injury, the animals received ventilation at 30% O_2 for 15 minutes before being transferred to the same chamber filled with 100% O_2 at 1 ata and remaining there for 4 hours. The animals were not restrained or anesthetized while in the chamber.

Cognition Testing

Morris Water Maze. The MWM test was performed as a measure of cognitive function following an acquisition paradigm on postinjury Days 11 to 15.[20] Briefly, the rats underwent four trials per day for 5 consecutive days. On each trial, the rats were placed in the pool at one of the four start locations (south, west, north, and east) in a randomized fashion. The animal's movements within the tank were recorded and analyzed with a video tracking system (Polytrack 4, San Diego Instruments). This tracking equipment allowed for the analysis of both the time to reach the goal platform and the swim speed. The time to find the platform was the primary dependent variable for the assessment of cognitive performance. The swim speed was calculated to ensure that the cognitive performance was not confounded

by motor deficits that could adversely affect the swimming performance.

Stereological Cell Quantification. The number of neurons in the hippocampal subregions was determined with stereological methods by applying the Olympus image system CAST program. Every third coronal hippocampal section 180 μm apart was examined. Nissl-stained neurons in the CA1, CA2/3, and hilar regions were counted within the counting frame, ignoring the cells in the upper- and lowermost focal planes while focusing through the thickness of the sections to avoid oversampling errors.[10]

Quantification of ATP

To examine the early effects of hyperoxia on tissue metabolism, ATP was measured 1 hour after treatment following a previously published protocol.[1] Fifteen minutes after injury, the rats received normobaric 30% O_2, normobaric 100% O_2, or HBO_2 treatment for 1 hour. At the end of the treatment, while the animal was under deep anesthesia, the skull was opened and the brain was exposed between the lambdoid and coronal sutures, and the parietal ridges. The neocortical tissue containing the site of injury was quickly removed and dropped into liquid N_2. The animal was then killed while under the same deep anesthesia.

Acid Extraction. Each sample was removed from the liquid N_2 and, while still frozen solid, was quickly crushed with a pestle and weighed. The tissues (20 to 50 mg) were then placed in 75 × 10–mm glass tubes containing 0.4 ml of 12% trichloroacetic acid and homogenized on ice using a custom glass homogenizer for 30 minutes. At the end of 30 minutes, the tubes were centrifuged for 10 minutes at 3000 G at 10°C, and the supernatants were transferred to clean tubes. To neutralize the samples, a 3:1 mixture of R13 Freon and tri-n-octylamine was made and applied in a fume hood. Next, 0.8 ml of the mix was added to the trichloroacetic acid extract and mixed on a vortexer for 2 minutes to combine the phases. The samples were centrifuged at 3000 G for 10 minutes at 10°C to separate the phases. The upper layer containing the neutralized extract was placed in a microcentrifuge tube, snap frozen in liquid N_2, and stored at −80°C until analyzed. The Freon/tri-n-octylamine mix was absorbed into activated charcoal and discarded in accordance with Environmental Protection Agency rules.

High-Performance Liquid Chromatography System. The measurement of adenine nucleotides and NAD+ was performed using HPLC. The HPLC standards containing known amounts of adenine nucleotides, nucleosides, purine bases, and NAD+ or unknown samples were injected into a Waters Intelligent Sample Processor HPLC system (model 710B, Waters Associates). The system included an Alltech 101 solvent selector (Alltech Association) coupled to a Waters 6000A solvent delivery system. Separation of the metabolites was achieved using a NOVA-Pak-A C18 column (10 × 8 mm, 5-μm particle sizes) inside a Waters Radial Compress Module 100. Metabolites were measured and quantified using a Waters 490 four-channel absorbance detector at a wavelength of 254 nm coupled to a Waters 730 integrator.

Separation Using HPLC. Step-gradient elution was performed using a three-solvent protocol with a flow rate of 1.5 ml/minute. High-performance liquid chromatography–grade ammonium phosphate (100 mmol/L) buffered at pH 4.7 allowed the sequential elution of ATP, ADP, hypoxanthine, xanthine, and AMP. The second solvent, 7% methanol, eluted NAD+. The third solvent, 40% methanol, allowed the final elution of inosine and adenosine. All of the data were expressed as nanomoles per gram tissue wet weight.

Histological Studies

A randomly selected subset of animals (four animals/group) was used in a histological analysis. At 21 days postinjury, the animals were deeply anesthetized and transcardially perfused with 150 ml of phosphate-buffered saline followed by 150 ml of 4% paraformaldehyde. The brains were removed from the skull and fixed in 4% paraformaldehyde at 4°C for 48 hours. A small wedge (remote from the hippocampus) in the left hemisphere was made with a dissecting blade to ensure accurate recognition of the left and right hemispheres for subsequent tissue processing. Coronal 60-μm sections throughout the entire length of the hippocampus were cut using a vibratome (VT1000s, Leica). Every third section was mounted on a microscope slide (Superfrost/Plus) and stained for Nissl substance with cresyl violet, for a total of 10 sections per brain.

Mitochondrial Free Radical Generation

To determine whether injury and O_2 treatment induced free radical formation, the oxidation-sensitive dye, DHR (Molecular Probes) was used to measure the relative mitochondrial ROS generation.[4] This lipophilic, mitochondria-specific dye is oxidized by peroxynitrite and peroxide to form rhodamine 123, which can fluoresce and thus be detected. For rhodamine 123 fluorescence measurement, mitochondria-enriched samples were prepared following a previously published method.[48] Briefly, after completing treatment, the animals were perfused with ice-cold phosphate-buffered saline, and the neocortex under the craniotomy was removed and homogenized in an extracting buffer (0.32 mol/L sucrose, 20 mmol/L HEPES, and 2 mmol/L dipotassium ethylenediaminetetraacetic acid, pH 7.2). The homogenate was centrifuged twice at 2000 G, and the supernatant was collected. After further centrifugation at 11,600 G two times, the pellet was collected, resuspended in buffer, and stored at −80°C until used. Approximately 60 μg of the mitochondria-enriched fraction was incubated with the DHR for 30 minutes at 37°C, for a final concentration of 5 μmol/L of the mitochondria-enriched fraction. The samples were then centrifuged at 11,200 G and washed twice in DHR-free buffer. The pellets were then resuspended in extracting buffer and 100-μl aliquots were transferred to a Nunc 96-well uncoated black plate for fluorescence quantification on a BMG FLUOstar Galaxy plate reader (485 nm excitation and 520 nm emission, fluorescence). All samples were run in duplicate, and ROS generation was expressed as relative levels of rhodamine 123 per microgram protein. Because rhodamine 123 measures only the production of peroxide and peroxynitrite, this methodology measured only a small portion of the entire free radical generation spectrum.

Statistical Analysis

The MWM performances were analyzed using a 4 (number of groups) × 5 (number of days) split-plot ANOVA. To determine specific group differences, the Duncan multiple range test was used. The ATP data were evaluated using an ANOVA, and a post hoc Student t-test was applied to compare the group means. A probability value less than 0.05 was considered statistically significant in our analysis of the MWM and ATP data. Stereological data were evaluated using an ANOVA followed by a post hoc Student t-test for comparing the group means. In all these analyses, a probability value less than or equal to 0.05 was considered statistically significant.

All data are expressed as the means ± SEM for each group.

Results

Measurements of ATP

To examine the early effect of hyperoxia treatment on mitochondrial function following TBI, ATP was extracted and measured using HPLC. We found significant reductions in cerebral ATP levels in injured animals (lateral FPI) compared with those in sham-injured animals ($p \leq 0.05$). There were also corresponding significant reductions in ADP and NAD+ levels in these same animals ($p \leq 0.05$). The AMP levels were slightly increased in injured animals on 30% O_2 compared with sham-injured animals (Fig. 1). When examining the effect of 1 hour of hyperoxia treatment, ATP levels were significantly elevated in both the normobaric 100% O_2 and the HBO_2-treated groups compared with levels in injured animals that had received 30% O_2. Both hyperoxia treatments increased ATP to levels near that in the sham-injured rats ($p \leq 0.05$). The AMP levels were significantly reduced in injured animals that had received either hyperoxic treatment when compared with the

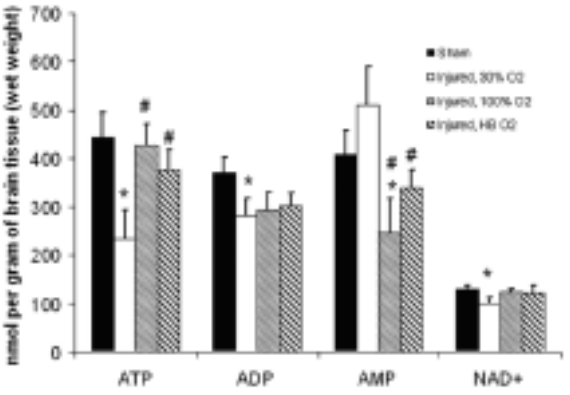

FIG. 1. Bar graph demonstrating cerebral ATP, ADP, AMP, and NAD+ levels 1 hour after FPI and hyperoxia treatment. The ATP level in injured animals breathing 30% O_2 is significantly reduced compared with levels in sham-injured animals (*$p \leq 0.05$, four rats from each treatment group). The ATP level is significantly increased in injured animals treated with hyperoxia compared with the level in animals breathing 30% O_2 (#$p \leq 0.05$, four rats from each treatment group). The ADP level is significantly reduced in injured animals breathing 30% O_2 compared with the level in sham-injured animals (*$p \leq 0.05$). The AMP level is also significantly reduced in the 100% O_2–treated group compared with that in sham-injured animals (*$p \leq 0.05$) and is significantly reduced in both the HBO_2- and the 100% O_2–treated groups compared with that in the animals breathing 30% O_2 (#$p \leq 0.05$). The NAD+ level is significantly reduced in the injured animals breathing 30% O_2 compared with that in the sham-injured animals (*$p \leq 0.05$).

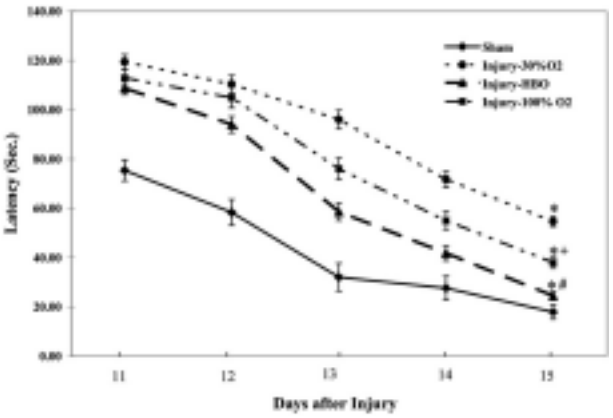

FIG. 2. Line graph depicting the effect of O_2 treatment on MWM performance following TBI. The MWM test was performed on Days 11 to 15 post-FPI. All three injured animal groups had a significantly longer latency period compared with the sham-injured animals (*$p \leq 0.05$, 10 rats for each group). The HBO_2-treated group took a significantly shorter time to find and mount the hidden platform than the 100% O_2– and 30% O_2–treated groups of injured animals on all days tested (#$p \leq 0.05$). The 100% O_2–treated group had a significantly shorter latency compared with the 30% O_2–treated group of injured animals (+$p \leq 0.05$). Data are expressed as the means ± SEMs.

animals that had received 30% O_2 ($p \leq 0.05$). The ADP and NAD+ levels in both hyperoxia-treated groups were slightly higher than those in the 30% O_2–treated group and were similar to those in the sham-injured animals.

Morris Water Maze Assessment

At 11 to 15 days postinjury, cognitive deficits were tested using the MWM. The results showed that the injured animals that received only 30% O_2 (mean time to reach the platform 90.5 seconds) took a significantly longer time to reach the goal compared with rats in the other groups (100% O_2 treatment, mean time 77.4 seconds; HBO_2 treatment, mean time 65.5 seconds; and sham-injured group treated with 30% O_2, mean time 42.2 seconds). Similarly, the injured animals treated with normobaric 100% O_2 took a longer time to find the goal than the injured animals treated with HBO_2. Moreover, the injured animals treated with HBO_2 took a longer time than the sham-injured group but less time than the other two treatment groups to find the goal (Fig. 2). The ANOVA analysis of goal latencies revealed a significant main effect of group [$F(3,54) = 48.7$, $p \leq 0.001$], day [$F(4,216) = 445.6$, $p \leq 0.01$], and group × day [$F(12,216) = 6.5$, $p \leq 0.001$].

Histological Study

Four rat brains in each group were randomly picked to evaluate neuronal cell loss in the hippocampus. Cresyl violet stains intact neurons. Those neurons undergoing early degeneration would have fully degenerated and been removed by 21 days after injury. Neurons in the CA1, CA2/3, and hilar regions in the ipsilateral hippocampus were counted using an unbiased stereological method. We found no significant cell loss in the CA1 area in all three injured rat groups compared with that in the sham-injured group (data not shown). In the CA2/3 area, all injured groups had significant cell loss compared with that in the sham-injured animals ($p \leq 0.05$). However, animals that had been treated with HBO_2 demonstrated significantly less neuronal cell loss compared with that in animals that had received normobaric 30% or normobaric 100% O_2 treatment only ($p \leq 0.05$; Fig. 3). In addition, there was no significant difference in neuronal cell counts between animals that received 30% O_2 and those that received 100% O_2. In the hilar region, all injured animals had significant neuronal cell loss as compared with that in the sham-injured group ($p \leq 0.05$; Fig. 4). However, injured animals treated with HBO_2 had significantly less neuronal cell loss compared with that in injured animals treated with 30% O_2 ($p \leq 0.05$). There was no significant difference among injured groups treated with 30% O_2 and those treated with 100% O_2.

Mitochondrial Free Radical Formation

The ability of the mitochondria to produce ROS, specifically peroxide and peroxynitrite, was not significantly altered in injured rats compared with sham-injured controls at 1 or 4 hours, as measured by rhodamine 123 fluorescence. Likewise, no significant changes in peroxide and peroxynitrite were demonstrated based on treatment with 30%, 100% O_2, or HBO_2 plus 100% O_2 at 1 or 4 hours in both sham-injured and injured animals (Table 1).

Discussion

The use of HBO_2 to treat TBI remains controversial. Conversely, its effectiveness has been shown for conditions such as decompression sickness, anaerobic infections, tis-

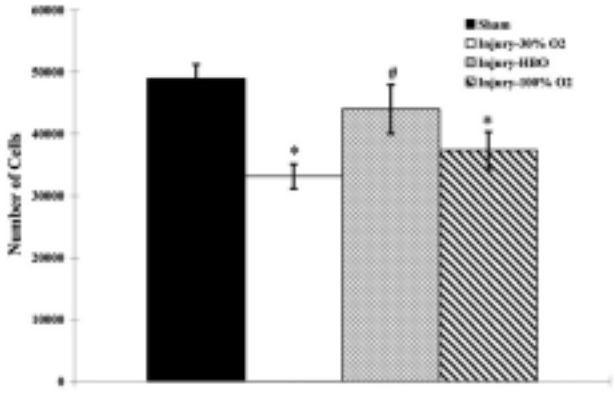

FIG. 3. Bar graph demonstrating neuronal cell counts in the CA2/3 region of the ipsilateral hippocampus. Both 100% O_2– and 30% O_2–treated groups of injured animals had significant neuronal cell loss compared with the sham-injured group (*$p \leq 0.05$). The HBO_2-treated group had significantly less neuronal cell loss compared with both the 100% O_2– and 30% O_2–treated groups of animals (#$p \leq 0.05$). Data are expressed as the means ± SEMs.

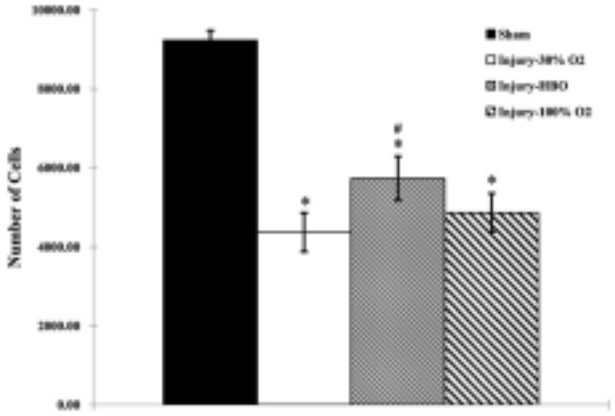

FIG. 4. Bar graph showing neuronal cell counts in the hilar region of the ipsilateral hippocampus. All three injured animal groups had significant neuronal cell loss compared with the sham-injured group (*$p \leq 0.05$). The HBO_2-treated group had significantly less neuronal cell loss than the 30% O_2–treated injured animals (#$p \leq 0.05$). Data are expressed as the means ± SEMs.

sue hypoxia, and diabetic wound healing.[23,28,37] In a landmark phase IIB controlled study, Rockswold et al.[42] showed improved outcome and lower ICP in a group of 168 patients with severe TBI treated with HBO_2 every 8 hours for up to 14 days after injury. Nevertheless, the widespread adoption of HBO_2 for the treatment of TBI is not established. We have shown that normobaric hyperoxia (100% fraction of inspired O_2 for 24 hours) was beneficial in two case–control cohort studies. Yet, the mechanisms for these beneficial effects remain poorly understood.[35,36] In the present study, we demonstrated that HBO_2 treatment in the rat, even for only 1 hour after moderate experimental FPI, can preserve cerebral ATP levels, improve cognitive recovery, and reduce hippocampal neuronal cell loss, thus providing experimental data to validate the efficacy of this treatment.

Effect of Hyperoxia on Cerebral Metabolism

After TBI, compromised O_2 delivery can result in impaired mitochondrial respiration, leading to a shift from aerobic to anaerobic metabolism with increased lactate production/accumulation and reduced ATP production.[7,26,39,45] Following TBI, a reduction in local blood flow caused by diverse mechanisms, such as microvessel compression, endovascular and perivascular edema, hypovolemic shock, and cerebral vasospasm, could contribute to this mitochondrial ATP reduction.[8,29,30,34] The acute decrease in the ATP/ADP ratio that arises from an increase in cytosolic ADP and a reduction in tissue ATP concentrations indicates a loss of energy production at a time when an increased demand for energy is occurring after acute brain injury.

In the current study, we focused our efforts on determining any adverse effect on mitochondrial ATP production early after TBI. We have shown that a moderate FPI can cause a significant reduction in cerebral ATP levels as early as 1 hour postinjury, indicating that mitochondrial function can be suppressed within this time frame, a finding consistent with data from other reports.[7,26,30] However, injured animals treated with either HBO_2 or 100% O_2 have shown significantly preserved mitochondrial function, as measured by cerebral ATP levels, compared with injured animals that received 30% O_2. The beneficial effects of HBO_2 and hyperoxia have thus already been proven in several clinical studies.[23,29,41,42,50] In a microdialysis study in which 52 patients with TBI had been treated with normobaric 100% O_2, reduced extracellular fluid lactate and increased extracellular fluid glucose were observed, suggesting that anaerobic glycolysis can be improved with normobaric 100% O_2 treatment.[49]

Rockswold and colleagues[41,42] have shown that HBO_2 treatment can improve aerobic metabolism in severely brain-injured patients by increasing the cerebral metabolic rate of O_2 and decreasing cerebrospinal fluid lactate levels. The regulation of the citric acid cycle by NADH may par-

TABLE 1
*Mitochondrial free radical formation**

Parameter	Group (no. of rats)			
	Sham-Injured	Injured	Injured	Injured
treatment	30% O_2	30% O_2	100% O_2	HBO_2
treatment time (hrs)				
1	978.6 ± 80.6 (4)	967.1 ± 48.7 (4)	952.1 ± 41.5 (6)	1001.7 ± 86.6 (4)
4	996.5 ± 17.8 (4)	979.8 ± 60.4 (5)	1014.2 ± 108.0 (4)	978.2 ± 24.8 (5)

* Values represent the relative rhodamine 123 fluorescence per microgram of protein ± SEM.

tially explain the effect of HBO_2 and hyperoxia. High concentrations of NADH have long been known to reduce the metabolic rate of the citric acid cycle.[24] Hyperbaric O_2 may lead to an increase in ATP production via the oxidation of mitochondrial NADH and consequently the production of NAD+ thereby donating electrons to the electron transport chain.[19] In turn, NAD+ has the ability to increase the functional rate of the citric acid cycle, thus further enhancing mitochondrial ATP generation.[24] In the present study, the levels of NAD+ in both HBO_2- and normobaric O_2–treated groups supports this phenomenon. However, the exact mechanisms of this increase in mitochondrial function due to enhanced tissue O_2 tension remain unclear and require further investigation.

Interestingly, we found in the current study that the ATP levels in the rats treated with normobaric hyperoxia (100% O_2) were higher than those in the HBO_2-treated group, although the difference did not reach statistical significance. This observation suggests that early treatment with normobaric O_2 may have beneficial effects on mitochondrial function in return for much less effort in comparison with HBO_2 treatment. In either case, what remains unclear is whether the preservation of cerebral ATP levels after TBI can be maintained for longer than 1 hour. We do know, however, that the administration of 100 mmol/L lactate over the course of 3 hours does result in a significant increase in ATP levels 3 hours after FPI and, because lactate increases mitochondrial O_2 consumption,[25] that there is likely to be a concomitant increase in ATP production. Nevertheless, further studies to determine the effect of hyperoxia treatment on the duration of cerebral ATP production after TBI are necessary.

Effect of Hyperoxia on Cognitive Function

Following FPI, mitochondrial damage can contribute to the progressive cell death observed in the parietotemporal cortex and the CA3 region of the hippocampus.[26] In the current study, we found that early HBO_2 treatment improves cognitive recovery and reduces neuronal cell loss in the hippocampus in rats following a moderate lateral FPI. In the MWM test, injured animals in all treatment groups (normobaric 30% O_2, normobaric 100% O_2, and HBO_2) took a significantly longer time to find the hidden platform as compared with the sham-injured animals; however, the HBO_2-treated animals had a significantly shorter latency than the injured animals in the other two treatment groups. These results demonstrated that HBO_2 treatment can improve cognitive recovery in rats following FPI. Nevertheless, injured animals treated with 100% O_2 alone also had a significantly shorter latency as compared with injured animals receiving 30% O_2. This finding implies that normobaric hyperoxia is also beneficial and is consistent with data in previous studies demonstrating that normobaric hyperoxia has a positive effect on brain-injured and ischemic animals.[39,46] Moreover, it is also consistent with data in clinical studies showing improvements in ICP and outcome after severe TBI in humans treated with normobaric hyperoxia.[49]

Effect of Hyperoxia on Hippocampal Cell Survival

Neuronal cell loss in the hippocampus has been suggested to be the major mechanistic link to cognitive impairment after experimental TBI.[17,20,43] In the current study, we quantified neuronal cell survival in the CA1, CA2/3, and hilar regions of the ipsilateral hippocampus at 21 days after injury so that uniform cell morphometry criteria could be applied; all lethally damaged neurons would be expected to have passed through all the states of necrotic or apoptotic cell death before this 21 day time point.[11] Such information is crucial, because many other therapeutic interventions in animal models of TBI have been focused on the extent of neuronal degeneration as an end point. In the areas of CA2/3 and the hilus, a significant decrease in the number of neurons was observed in all injured animals compared with the sham-injured animals. Note, however, that animals treated with HBO_2 had significantly less neuronal cell loss in both areas. This result clearly demonstrates that HBO_2 treatment can reduce neuronal cell loss in the hippocampus, which contributes to the improved cognitive recovery observed in the MWM performance.

Our data suggest that HBO_2 treatment has a greater beneficial effect on the injured animals than normobaric 100% O_2 alone. The positive results with normobaric 100% O_2 imply an incremental effect rather than an all or nothing effect. Using the same lateral FPI model, Daugherty and associates[13] have documented that HBO_2 produces brain tissue PO_2 levels 10 times greater than those induced by normobaric 30% O_2 (30 mm Hg compared with 300 mm Hg) and three times greater than those generated by normobaric 100% O_2 (100 mm Hg compared with 300 mm Hg). Rockswold and colleagues have demonstrated the same differential brain tissue PO_2 levels in humans (unpublished data). Note that HBO_2 at 1.5 ata increases the amount of dissolved O_2 in the plasma from 0.3 ml/dl in air to 3.2 ml/dl, which is on the order of 10 times. Given that the macrocirculation as well as the microcirculation can act as diffusion barriers to O_2 delivery to brain tissue, the brain tissue PO_2 levels achieved may be critical to mitochondrial function. Hyperbaric O_2 greatly increases the O_2 diffusion gradient from the lungs to blood to brain tissue. Nonhemoglobin O_2 transport may be more significant than previously thought. If, as it appears, the presence of O_2 induces the mitochondria to begin to function, brain tissue PO_2 levels may be critical. This finding could well explain the more robust effect of HBO_2 in the model of lateral FPI to the brain compared with normobaric 100% O_2.

Effect of Hyperoxia on Mitochondrial Free Radical Production

Data from several published reports attest to various increases in O_2 free radical end products after injury.[5,25,38] In most of these studies, however, high pressures (for example, > 3.0 ata) have been used. Observed differences may also be due to the selectivity of the dye and sample preparation. In the present study, the lack of significant alterations in rhodamine 123 fluorescence by HBO_2 or normobaric 100% O_2 treatment compared with 30% O_2 in injured animals suggests that the ability of mitochondria to generate O_2 free radicals may not be significantly enhanced by either treatment. This result supports our previous findings that hyperoxia does not significantly enhance tissue hydroxyl radical production, as determined by the salicylate trapping method.[15] In another study, free radical formation was not increased in normal brain with HBO_2 treatment at

3 ata, unless 100% O_2 was added for a sufficiently long period,[16] which suggests a possible free radical formation threshold. Therefore, the combination of very high hyperbaric pressures and the prolonged use of hyperoxia may limit the usefulness of chamber pressures for HBO_2 treatment with 100% O_2; hence, the use of 1.5 ata is seen as a major advantage. Dihydrorhodamine 123 is sensitive to the production of peroxide and peroxynitrite but not to superoxide (O_2^-) or nitric oxide. Therefore, the current findings do not exclude the possibility of increasing production of other radicals or of free radical generation from other important cellular sources other than mitochondria, for example, from the arachidonic acid pathways in the cytosol.

Conclusions

The results of this study show that early hyperbaric and normobaric O_2 therapies have significant beneficial effects following head injury. Both normobaric 100% O_2 and HBO_2 increased ATP levels in the injured rat brain to levels close to those found in sham-injured animals. Neither hyperbaric nor normobaric O_2 treatment significantly affected the ROS peroxide and peroxynitrite in sham-injured and injured rats. However, HBO_2-treated injured rats showed a significantly shorter latency on the MWM test than the injured rats treated with 100% O_2 alone. Moreover, the combined treatments of 100% O_2 and HBO_2 significantly preserved more hippocampal neuronal cells in the CA2/3 and hilar regions than 100% O_2 alone after injury. These results provide a mechanistic basis for both of these treatment modalities and support the findings of previous studies in humans. Additional studies to isolate the enzymatic and biochemical sites of action of increased O_2 tension within the mitochondria are needed and warrant further clinical trials with hyperoxia, with and without HBO_2.

Acknowledgment

We gratefully acknowledge the expert technical assistance of Dr. Anwar Abd-Elfattah for the ATP analysis by HPLC.

References

1. Abd-Elfattah AS, Wechsler AS: Superiority of HPLC to assay for enzymes regulating adenine nucleotide pool intermediates metabolism: 5′-nucleotidase, adenylate deaminase, adenosine deaminase and adenylosuccinate lyase—a simple and rapid determination of adenosine. **J Liq Chromatogr 10:**2653–2694, 1987
2. Alves OL, Daugherty WP, Rios M: Arterial hyperoxia in severe head injury: a useful or harmful option? **Curr Pharm Des 10:** 2163–2176, 2004
3. Auer RN, Wieloch T, Olsson Y, Siesjo BK: The distribution of hypoglycemic brain damage. **Acta Neuropathol 64:**177–191, 1984
4. Azbill RD, Mu X, Bruce-Keller AJ, Mattson MP, Springer JE: Impaired mitochondrial function, oxidative stress and altered antioxidant enzyme activities following traumatic spinal cord injury. **Brain Res 765:**283–290, 1997
5. Bochicchio M, Latronico N, Zani DG, Mariotti M, Morandini L, Acquarolo AM, et al: Free radical-induced lipoperoxidation and severe head injury. A clinical study. **Intensive Care Med 16:** 444–447, 1990
6. Bouma GJ, Muizelaar JP, Stringer WA, Choi SC, Fatouros P, Young HF, et al: Ultra early evaluation of regional cerebral blood flow in severely head-injured patients using xenon-enhanced computerized tomography. **J Neurosurg 77:**360–368, 1992
7. Buczek M, Alvarez J, Azhar J, Zhou Y, Lust WD, Selman WR, et al: Delayed changes in regional brain energy metabolism following cerebral concussion in rats. **Metab Brain Dis 17:**153–167, 2002
8. Bullock R, Maxwell WL, Graham DI, Teasdale GM, Adams JH: Glial swelling following human cerebral contusion: an ultrastructural study. **J Neurol Neurosurg Psychiatry 54:**427–434, 1991
9. Chesnut RM, Marshall SB, Piek J, Blunt BA, Klauber MR, Marshall LF, et al: Early and late systemic hypotension as a frequent and fundamental source of cerebral ischemia following severe brain injury in the Traumatic Coma Data Bank. **Acta Neurochir Suppl (Wien) 59:**121–125, 1993
10. Coggeshall RE, Lekan HA: Methods for determining numbers of cells and synapses: a case for more uniform standards of review. **J Comp Neurol 364:**6–15, 1996
11. Conti AC, Raghupathi R, Trojanowski JQ, McIntosh TK: Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period. **J Neurosci 18:** 5663–5672, 1998
12. Cortez SC, McIntosh TK, Noble LJ: Experimental fluid percussion brain injury: vascular disruption and neuronal and glial alterations. **Brain Res 482:**271–282, 1989
13. Daugherty WP, Levasseur JE, Sun D, Rockswold GL, Bullock MR: Effects of hyperbaric oxygen therapy on cerebral oxygenation and mitochondrial function following moderate lateral fluid-percussion injury in rats. **J Neurosurg 101:**499–504, 2004
14. Dixon CE, Lyeth BG, Povlishock JT, Findling RL, Hamm RJ, Marmarou A, et al: A fluid percussion model of experimental brain injury in the rat. **J Neurosurg 67:**110–119, 1987
15. Doppenberg EM, Rice MR, Di X, Young HF, Woodward JJ, Bullock R: Increased free radical production due to subdural hematoma in the rat: effect of increased inspired oxygen fraction. **J Neurotrauma 15:**337–347, 1998
16. Elayan IM, Axley MJ, Prasad PV, Ahlers ST, Auker CR: Effect of hyperbaric oxygen treatment on nitric oxide and oxygen free radicals in rat brain. **J Neurophysiol 83:**2022–2029, 2000
17. Graham DI: Hypoxia and vascular disorder, in Adams JH, Duchen LW (eds): **Greenfield's Neuropathology, ed 5.** New York: Oxford University Press, 1992, pp 153–268
18. Graham DI, Ford I, Adams JH, Doyle D, Teasdale GM, Lawrence AE, et al: Ischemic brain damage is still common in fatal non-missile head injury. **J Neurol Neurosurg Psychiatry 52:**346–350, 1989
19. Hamm RJ: Neurobehavioral assessments of outcome following traumatic brain injury in rats: evaluation of selected measures. **J Neurotrauma 18:**1207–1216, 2001
20. Henderson LM, Chappell JB: Dihydrorhodamine 123: a fluorescent probe for superoxide generation? **Eur J Biochem 217:** 973–980, 1993
21. Jain KK: **Textbook of Hyperbaric Medicine, ed 4.** Kirklan, WA: Hogrefe & Huber, 2004, pp 264–268
22. Kawamura S, Yasui N, Shirasawa M, Fukasawa H: Therapeutic effects of hyperbaric oxygenation on acute focal cerebral ischemia in rats. **Surg Neurol 34:**101–106, 1990
23. Kessler L, Bilbault P, Ortega F, Grasso C, Passemard R, Stephan D, et al: Hyperbaric oxygenation accelerates the healing rate of nonischemic chronic diabetic foot ulcers: a prospective randomized study. **Diabetes Care 26:**2378–2382, 2003
24. Lehninger AL: The citric acid cycle, in Anderson S, Fox J (eds): **Principles of Biochemistry.** New York: Worth Publishers, Inc., 1982, pp 435–466
25. Levasseur JE, Alessandri B, Reinert M, Clausen T, Zhou Z, Altememi N, et al: Lactate, not glucose, up-regulates mitochondrial oxygen consumption both in sham and lateral fluid percussed rat brains. **Neurosurgery 59:**1122–1131, 2006
26. Lewen A, Matz P, Chen PH: Free radical pathways in CNS injury. **J Neurotrauma 17:**871–890, 2000
27. Lifshitz J, Friberg H, Neumar RM, Raghupathi R, Welsh FA, Janmey P, et al: Structural and functional damage sustained by mitochondria after traumatic brain injury in the rat: evidence for dif-

ferentially sensitive population in the cortex and hippocampus. **J Cereb Blood Flow Metab 23:**219–231, 2003
28. Lowenstein DH, Thomas MJ, Smith DH, McIntosh TK: Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. **J Neurosci 12:**4846–4853, 1992
29. Mader JT, Adams KR, Wallace WR, Calhoun JH: Hyperbaric oxygen as adjunctive therapy for osteomyelitis. **Infect Dis Clin North Am 4:**433–440, 1990
30. Martin NA, Patwardhan RV, Alexander MJ, Africk CZ, Lee JH, Shalmon E, et al: Characterization of cerebral hemodynamic phases following severe head trauma: hypoperfusion, hyperemia, and vasospasm. **J Neurosurg 87:**9–19, 1997
31. Mautes AE, Thome D, Steudal W, Nacimiento AC, Yang Y, Shohami E: Changes in regional energy metabolism after closed head injury in the rat. **J Mol Neurosci 16:**33–39, 2001
32. McIntosh TK, Smith DH, Voddi M, Perri BR, Stutzmann JM: Riluzole, a novel neuroprotective agent, attenuates both neurologic motor and cognitive dysfunction following experimental brain injury in the rat. **J Neurotrauma 13:**767–780, 1996
33. McIntosh TK, Vink R, Noble L, Yamakami I, Fernyak S, Soares H, et al: Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. **Neuroscience 28:**233–244, 1989
34. Menon DK, Cole J, Gupta AK, Fryer TD, Smielewski P, Chatfield DA, et al: Diffusion limited oxygen delivery following head injury. **Crit Care Med 32:**1384–1390, 2004
35. Menzel M, Doppenberg EM, Zauner A, Soukup J, Reinert MM, Bullock R: Increased inspired oxygen concentration as a factor in improved brain tissue oxygenation and tissue lactate levels after severe human head injury. **J Neurosurg 91:**1–10, 1999
36. Menzel M, Doppenberg EM, Zauner A, Soukup J, Reinert MM, Clausen T, et al: Cerebral oxygenation in patients after severe head injury: monitoring and effect of arterial hyperoxia on cerebral blood flow, metabolism and intracranial pressure. **J Neurosurg Anesthesiol 11:**240–251, 1999
37. Muehlberger PM, Pilmanis AA, Webb JT, Olson JE: Altitude decompression sickness symptom resolution during descent to ground level. **Aviat Space Environ Med 75:**496–499, 2004
38. Nishio S, Yunoki M, Noguchi Y, Kawauchi M, Asari S, Ohmoto T: Detection of lipid peroxidation and hydroxyl radicals in brain contusion of rats. **Acta Neurochir Suppl 70:**84–86, 1997
39. Palzur E, Vlodavsky E, Mulla H, Arieli R, Feinsod M, Soustiel JF: Hyperbaric oxygen therapy for reduction of secondary brain damage in head injury: an animal model of brain contusion. **J Neurotrauma 21:**41–48, 2004
40. Robertson CS, Valadka AB, Hannay HJ, Contant CF, Gopinath SP, Cormio M, et al: Prevention of secondary ischemic insults after severe head injury. **Crit Care Med 27:**2086–2095, 1999
41. Rockswold GL, Ford SE, Anderson DC, Bergman TA, Sherman RE: Results of a prospective randomized trial for treatment of severely brain-injured patients with hyperbaric oxygen. **J Neurosurg 76:**929–934, 1992
42. Rockswold SB, Rockswold GL, Vargo JM, Erickson CA, Sutton Rl, Bergman TA, et al: Effects of hyperbaric oxygenation therapy on cerebral metabolism and intracranial pressure in severely brain injured patients. **J Neurosurg 94:**403–411, 2001
43. Schmidt-Kastner R, Freund TF: Selective vulnerability of the hippocampus in brain ischemia. **Neuroscience 40:**599–636, 1991
44. Signoretti S, Marmarou A, Tavazzi B, Lazzarino G, Beaumont A, Vagnozzi R: N-Acetylaspartate reduction as a measure of injury severity and mitochondrial dysfunction following diffuse traumatic brain injury. **J Neurotrauma 18:**977–991, 2001
45. Singhal AB, Dijkhuizen RM, Rosen BR, Lo EH: Normobaric hyperoxia reduces MRI diffusion abnormalities and infarct size in experimental stroke. **Neurology 58:**945–952, 2002
46. Smith DH, Okiyama K, Thomas MJ, Claussen BS, McIntosh TK: Evaluation of memory dysfunction following experimental brain injury using the Morris Water Maze. **J Neurotrauma 8:**259–269, 1991
47. Springer JE, Azbill RD, Carlson SL: A rapid and sensitive assay for measuring mitochondrial metabolic activity in isolated neural tissue. **Brain Res Brain Res Protoc 2:**259–263, 1998
48. Squire LR, Zola-Morgan S: The medial temporal lobe memory system. **Science 253:**1380–1386, 1991
49. Tolias CM, Reinert M, Seiler R, Gilman C, Scharf A, Bullock MR: Normobaric hyperoxia-induced improvement in cerebral metabolism and reduction in intracranial pressure in patients with severe head injury: a prospective historical cohort-matched study. **J Neurosurg 101:**435–444, 2004
50. Zamboni WA, Browder LK, Martinez J: Hyperbaric oxygen and wound healing. **Clin Plast Surg 30:**67–75, 2003

Manuscript submitted July 29, 2005.
Accepted October 3, 2006.
This study was funded by the National Institutes of Health Grant No. NS12587 and the Reynolds Foundation (M.R.B.).

Current address for Dr. Daugherty: Department of Neurosurgery, Mayo Clinic, Rochester, Minnesota.
Address reprint requests to: M. Ross Bullock, M.D., Ph.D., Department of Neurosurgery, Medical College of Virginia, Virginia Commonwealth University, 1200 East Broad Street, West Hospital 8 South, Richmond, Virginia 23298-0631. email: Mbullock@mcvh-vcu.edu.