

Synthes Award for Resident Research on Brain and Craniofacial Injury: Normoxic Ventilatory Resuscitation After Controlled Cortical Impact Reduces Peroxynitrite-Mediated Protein Nitration in the Hippocampus

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ABSTRACT

Resuscitation with 100% ventilatory oxygen is routinely initiated after severe traumatic brain injury (TBI). Despite the objective to improve oxygenation of the injured brain, there are concerns about the increased production of reactive oxygen species (ROS), which can lead to further neuronal damage. 3-nitrotyrosine (3-NT), the product of peroxynitrite-mediated tyrosine residue nitration, has been used as a marker for ROS-induced oxidative damage to proteins. We hypothesized that posttraumatic resuscitation with hyperoxic ventilation with a fraction of inspired oxygen (F_{iO_2} , 100%) results in increased ROS-induced damage to proteins compared with resuscitation with normoxic ventilation or room air (F_{iO_2} , 21%).

Male Sprague-Dawley rats underwent controlled cortical impact (CCI) and were resuscitated with either normoxic or hyperoxic ventilation for 1 hour after injury ($n = 5$ per group). Sham-operated control groups received 1 hour of normoxic or hyperoxic ventilation without CCI ($n = 4-5$ per group). Twenty-four hours after injury, rats were perfused with fixative, and hippocampi were evaluated for levels of 3-NT immunostaining. In a second experiment, for a delayed assessment of neuronal survival, another set of rats similarly underwent CCI and normoxic or hyperoxic ventilation for 1 hour ($n = 4$ per group), and a sham-operated group was used as a control ($n = 4$). One week after injury, neuronal cell counts and abnormal cell quantification were performed after staining with the neuron-specific NeuN antibody.

Quantification of 3-NT staining revealed significantly increased levels in the ipsilateral hippocampus in the hyperoxic CCI group. The normoxic group showed a 51.0% reduction of staining in CA1 when compared with those rats resuscitated with hyperoxia and a 50.8% reduction in CA3

(both $P < 0.05$). There was no significant difference in staining between the injured normoxic group and the sham-operated groups. In the delayed analysis of neuronal survival, although neuronal counts were reduced in the hippocampus on the injured side in both injured groups, there was no significant difference between hyperoxic and normoxic groups. Similarly, abnormal cell counts were not significantly different between groups.

In this clinically relevant model of TBI, normoxic resuscitation significantly reduced levels of oxidative damage to proteins compared with hyperoxic resuscitation. Delayed neuronal counts showed no beneficial effect of hyperoxic resuscitation. These findings indicate that hyperoxic ventilation in the early stages after severe TBI may exacerbate oxidative damage to proteins. Future studies should examine the relationship between protein oxidation and histologic and neurologic outcome in TBI.

Improvement of cerebral oxygenation after human TBI is one of the main therapeutic goals in the attempt to prevent secondary brain injury. After severe TBI, there is cerebral edema and often decreased cerebral blood flow (CBF), which causes a shift from aerobic to anaerobic metabolism. The presence of impaired oxidative metabolism is suggested by increased brain tissue lactate levels after TBI and reduction of lactate levels with increased inspired oxygen concentration (F_{iO_2} 100%).³⁰ In fact, in the prehospital setting, endotracheal intubation and initial resuscitation with an F_{iO_2} of 100% is routinely used in patients after severe TBI. In an effort to prevent hypoxia and secondary brain injury, it is recommended as a guideline to avoid oxygen saturation $<90\%$ in the field or a $Pa_{O_2} <60$ mm Hg.⁶ However, often large concentrations of oxygen are continually given during resuscitation and throughout the first 24 hours after injury, which results in Pa_{O_2} levels well above physiologic conditions.

Despite the seemingly beneficial effect of increased F_{iO_2} or normobaric hyperoxia on cerebral metabolism after

TBI,⁴⁴ the potential adverse effects must be considered. First, the generation of ROS occurs by a reaction without saturation at the inner mitochondrial membrane in the presence of large concentrations of oxygen. These products can result in oxidized proteins, lipids, DNA, and RNA, which can, in turn, impair cell metabolism and cell viability. Second, the harmful effects of hyperoxia on the lungs, including atelectasis, proinflammatory processes, fibrosis, and pulmonary hypertension have been well demonstrated.^{22,39} Underlying this issue, there is also a question of how much oxygen, when delivered at large concentrations to the lungs and blood stream, is actually effectively delivered to the brain tissue.^{23,28,38}

The aim of this study is to determine the effect of hyperoxic (Fio₂ 100%) versus normoxic (Fio₂ 21%) ventilatory resuscitation after TBI on the production of ROS by using an experimental model of CCI. Peroxynitrite is formed from a reaction between the ROS nitric oxide and superoxide. 3-NT is the product of peroxynitrite-mediated nitration of tyrosine residues and has been used as a marker for oxidative stress. We designed this study to determine the levels of 3-NT in animals treated with hyperoxic versus normoxic ventilatory resuscitation after CCI.

MATERIALS AND METHODS

The subjects were 31 male Sprague-Dawley rats with a mean age of 10.6 weeks (range, 9.6–12.3) and a mean weight of 326.7 g (range, 262–432). All experiments were performed in accordance with the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine.

Surgery

Rats were placed in a Plexiglas chamber, and anesthesia was induced by 4.5% inhaled isoflurane in pressurized room air for 2 to 3 minutes. They were then endotracheally intubated with a 14-gauge Angiocath and placed on positive pressure ventilation on a small animal ventilator (Harvard Apparatus, Holliston, MA). Maintenance anesthesia was delivered with 3% isoflurane. The animal was placed in a stereotactic frame. A rectal temperature probe and a heating blanket (Henry Schein, Melville, NY) were used to maintain

the body temperature at 37°C. The mean temperature at time of CCI was 36.9 ± 0.1°C (range, 34.4–37.7).

After a midline scalp incision, the left parietal bone was exposed. A left parietal craniotomy was performed with the aid of a surgical microscope and a dental drill (Fine Science, Foster City, CA). Then, the CCI device was calibrated with respect to the exposed dura within the craniotomy. The parameters of impact in injured rats were a depth of 1.5 mm, a mean velocity of 5.62 ± 0.04 m/s (range, 5.24–5.88), and a duration of 50 ms. After impact, the hyperoxic rats were ventilated with 100% O₂ for 1 hour. Normoxic rats were continued on ventilation with room air (Fio₂ 21%) for 1 hour. The craniotomy site was covered with dental acrylic, and the scalp incision was closed with silk suture. Sham-operated groups received a craniotomy without CCI and were ventilated with either hyperoxia or normoxia for 1 hour. Arterial blood gas analysis was performed in all rats 30 minutes after injury with blood obtained from the tail artery. The PO₂, pH value, and PCO₂ of the groups analyzed for 3-NT are shown in *Table 45.1* and those analyzed for NeuN in *Table 45.2*. After 1 hour of resuscitation, the isoflurane was stopped. The rats were extubated after exhibiting spontaneous respiration and movement.

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3-NT

Twenty-four hours after injury, rats were injected with a lethal dose of intraperitoneal sodium pentobarbital. Rats were perfused first with intracardiac saline and then with 4% paraformaldehyde. The brains were removed and postfixed in a 2.5% acrolein (Polysciences, EM grade, Warrington, PA) solution in 4% paraformaldehyde, pH 6.8, for 1 hour, and kept in 4% paraformaldehyde for 1 day. The brains were then sliced to isolate 3-mm coronal blocks that included the hippocampi. These blocks were then transferred to a 30% sucrose solution and left until they sank. Using a freezing microtome, blocks were cut into 25-μm sections in a 1:6 series for staining. Sections were stored at -20°C in a cryoprotectant polyethylene glycol for at least 1 week before staining.⁴⁷

TABLE 45.1. Arterial blood gas analysis during ventilatory resuscitation after CCI for rats analyzed for 3-NT^{a,b}

Subject group	PO ₂	PCO ₂	pH value
Injured hyperoxic (n = 5)	396.5 ± 26.9 ^c	32.0 ± 3.6	7.51 ± 0.04
Injured normoxic (n = 5)	105.6 ± 4.3	31.4 ± 6.1	7.46 ± 0.06
Sham hyperoxic (n = 5)	346.0 ± 30.5 ^c	30.6 ± 2.6	7.52 ± 0.03
Sham normoxic (n = 4)	102.8 ± 8.7	33.4 ± 2.6	7.49 ± 0.03

^aValues are expressed as mean ± SE.

^bCCI, controlled cortical impact; 3-NT, 3-nitrotyrosine; PO₂, partial pressure of oxygen; PCO₂, partial carbon dioxide pressure.

^cP < 0.05 compared with normoxic group counterparts.

TABLE 45.2. Arterial blood gas analysis during ventilatory resuscitation after CCI for rats analyzed for NeuN^{a,b}

Subject group	PO ₂	PCO ₂	pH value
Injured hyperoxic (n = 4)	306.8 ± 25.6 ^c	35.4 ± 1.9	7.48 ± 0.02
Injured normoxic (n = 4)	128.1 ± 16.8	32.7 ± 4.3	7.50 ± 0.04
Sham hyperoxic (n = 4)	346.7 ± 28.7 ^c	34.2 ± 2.6	7.52 ± 0.03

^aValues are expressed as mean ± SE.

^bCCI, controlled cortical impact; NeuN, neuron-specific antibody; PO₂, partial pressure of oxygen; PCO₂, partial carbon dioxide pressure.

^cP < 0.05 compared with normoxic group counterparts.

Sections were then washed in 0.1 M of Tris buffer solution, pH 7.6, six times, 10 minutes each, to rinse out the cryoprotectant. They were then incubated in a 1% sodium borohydride solution for 20 minutes and rinsed multiple times with Tris buffer. The following staining method was adapted from a protocol previously described by Lorch et al.²⁶ Sections were mounted on glass slides and allowed to dry overnight. The next day, slides were rehydrated in Tris buffer and treated in a 5% H₂O₂/methanol solution for 30 minutes to kill endogenous peroxidase activity. They were then washed in tap water for 10 minutes. Slides were then submerged in a boiling citrate solution for 10 minutes and then allowed to soak in the hot solution for another 10 minutes. They were then washed in distilled H₂O for 5 minutes and Tris buffer three times, 5 minutes each. Slices were blocked with a 50% goat serum Tris buffer solution for 1 hour in a moist chamber. Next, slices were treated with a 5% goat serum Tris buffer solution with rabbit anti-3-nitrotyrosine antibody (1:500; Upstate, Waltham, MA) overnight at 4°C. Slices were then washed in Tris buffer two times, 5 minutes each, and treated with biotinylated goat anti-rabbit antibody in Tris buffer (1:1000) for 1 hour at room temperature in a moist chamber. After washing again in Tris buffer two times, 5 minutes each, slices were stained with Nickel-diaminobenzidine (Ni-DAB) chromogen using the VectorStain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Stained slices were analyzed for intensity of 3-NT staining. Quantitative analyses were performed with computer-assisted image analysis system, which consisted of a Nikon Eclipse 800 photomicroscope, a Retiga 1300 cooled CCD digital camera (Biovision Technologies, Inc., Exton, PA), and a Macintosh G4 computer with IP Spectrum software (Scientific Image Processing, Version: 3.9.3 r2, Mac OS 10.3.3; Scanalytics, Fairfax, VA). The total area of staining was determined in regions of the hippocampus under 40× magnification. For each subject, an equivalent slice through the hippocampus was analyzed in the CA1, CA 2/3, and

dentate gyrus (DG) regions in both sides ipsilateral and contralateral to the injury. For each region of the hippocampus, three fields under 40× magnification were analyzed. The stage of the microscope was adjusted so that the cell layer was centered in the field and was oriented horizontally in the captured image. The amount of staining was expressed as the average area occupied by the black reaction product, which represented 3-NT immunoreactivity within the microscopic field. For each image analysis, segmentation values were set to eliminate any background staining, and the total area of staining (in square micrometers) was determined. All slices were analyzed by the same examiner for consistency.

NeuN

For delayed analysis of neuronal survival 7 days after injury, rats were perfused and brain slices were prepared using the same method described above for the 3-NT antibody. After storing sections at -20°C in polyethylene glycol for at least 1 week, they were stained using a free-floating double label immunocytochemistry protocol, previously described by Hoffman et al.¹⁹ Briefly, sections were washed in 0.05 M of potassium phosphate buffered saline (KPBS) six times, 10 minutes each, to rinse out the cryoprotectant. They were then incubated in a sodium borohydride solution, as described above, and rinsed multiple times in KPBS. The slices were then incubated with the primary antibody, mouse monoclonal anti-NeuN (1:120,000; Chemicon, Temecula, CA) in KPBS with 0.4% Triton-X for 1 hour at room temperature and then for 24 hours at 4°C. Sections were then rinsed again in KPBS six times, 10 minutes each. They were incubated with the secondary antibody, biotinylated horse anti-mouse antibody (1:600) in KPBS with 0.4% Triton-X for 1 hour. After rinsing again in KPBS five times, 10 minutes each, sections were prepared with the VectorStain Elite ABC kit. Sections were then rinsed in KPBS three times, 5 minutes each, then 0.175M sodium acetate three times, 5 minutes each, and then KPBS three times, 5 minutes each. The sections were then placed into a Ni-DAB H₂O₂ chromogen solution (250 mg of Ni sulfate, 2 mg of DAB, and 8.3 μL of 3% H₂O₂ /10 L of 0.175 sodium acetate solution). Sections were left in solution for 10 minutes and staining was then terminated by transferring to the sodium acetate solution. Stained sections were then mounted on glass slides, dehydrated, and coverslipped with Histomount (Zymed Laboratories, South San Francisco, CA).

For neuronal quantification, the same computer-assisted image analysis system described above for 3-NT was used. For each subject, an equivalent section through the hippocampus was analyzed for neuronal counts in the CA1 and CA3 regions in both sides ipsilateral and contralateral to the injury. In each hippocampal region, two high-power fields under 40× magnification were analyzed. The number of normal neurons was determined. In addition, abnormal neu-

rons that exhibited fragmented, pyknotic, or absent nuclei were also counted. All slices were analyzed by the same examiner for consistency.

Statistics

One way analysis of variance (ANOVA, Student-Newman-Keuls method) was used for data analysis. All comparisons with $P < 0.05$ are considered significant. All values are expressed as mean \pm SEM.

RESULTS

Arterial Blood Gas Analysis

Mean PO_2 , PCO_2 , and pH values obtained from the tail arterial blood after 30 minutes of ventilatory resuscitation after CCI for the rats analyzed for 3-NT are shown in *Table 45.1*. In the 3-NT immunohistochemistry experiment, the mean PO_2 values for the hyperoxic ventilated rats were 396.5 ± 26.9 for the injured rats and 346.0 ± 30.5 for the sham-operated rats. These values were significantly increased when compared to the normoxic counterparts with PO_2 values of 105.6 ± 4.3 and 102.8 ± 8.7 , with ($P < 0.001$). Arterial blood gas analysis for rats analyzed for NeuN are shown in *Table 45.2*. Similarly, rats that received hyperoxic resuscitation had PO_2 values of 306.8 ± 25.6 for the injured rats and 346.7 ± 28.7 for the sham-operated rats. These values were significantly increased compared with the normoxic rats with a mean PO_2 of 128.1 ± 16.8 ($P < 0.001$). In both experiments, there was no significant difference in PCO_2 or pH values between groups.

Nitrotyrosine Analysis

In this experiment, rats were subject to one of four conditions: injury with hyperoxic resuscitation ($n = 5$), injury with normoxic resuscitation ($n = 5$), sham operation with hyperoxic resuscitation ($n = 5$), or sham operation with normoxic resuscitation ($n = 4$). To determine levels of oxidative stress, brain slices through the hippocampus were obtained 24 hours after CCI and stained with the 3-NT antibody (*Fig. 45.1*). Quantification of 3-NT staining in the hippocampus ipsilateral to the injured cortex is displayed in *Figure 45.2*. In the hippocampal region of CA1, rats resuscitated with normoxia showed a 51.0% reduction of staining when compared with those rats resuscitated with hyperoxia ($P < 0.05$). In CA3, normoxic rats showed a 50.8% reduction in staining when compared with hyperoxic rats ($P < 0.05$). Furthermore, there was increased staining in the CA1 and CA3 regions of the injured hyperoxic group when compared with equivalent regions of both sham-operated groups ($P < 0.05$). In contrast, there was no difference in staining in these regions among the injured normoxic group and both sham-operated groups. There was also no difference in the staining of the DG between all groups. Of note, the sham-operated hyperoxic group showed increased staining in all hippocam-

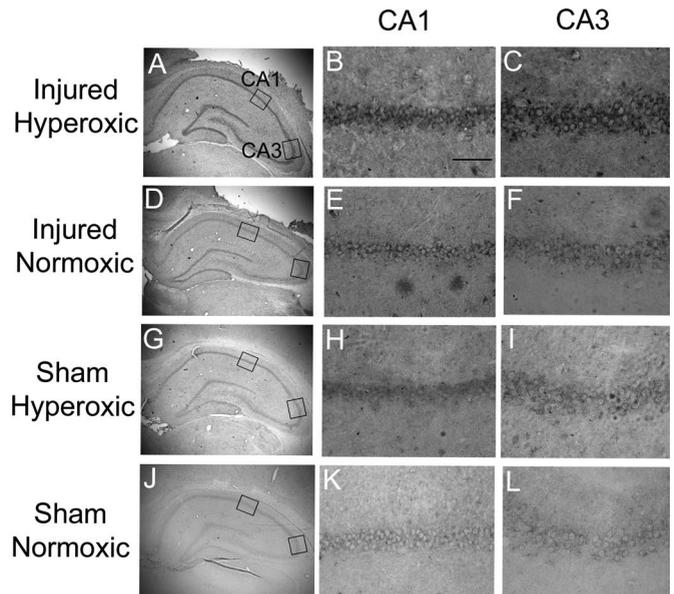


FIGURE 45.1 Sample photomicrographs of the hippocampus underlying the injured cortex with 3-NT (1:500) staining 24 hours after CCI for each of the subject groups: injured hyperoxic, A–C, injured normoxic, D–F, sham hyperoxic, G–I, and sham normoxic, J–L. Photomicrographs in the first column, A, D, G, and J, are low-power views of the hippocampus. The damaged cortex can be seen in both injured rats, A and D. Black boxes indicate the CA1 and CA3 regions, which are shown to the right at 40 \times magnification (CA1, B, E, H, and K, and CA3, C, F, I, and L). The injured hyperoxic group showed the most intense staining in the CA1 and CA3 regions of the hippocampus. The least intense staining was seen in the sham-operated normoxic group. Scale bar = 50 μ m.

pal regions compared with the sham-operated normoxic group, but there was no statistical significance ($P = 0.679$ in CA1; $P = 0.475$ in CA3; $P = 0.271$ in DG).

In the hyperoxic group, the hippocampus on the injured side of the brain demonstrated increased staining when compared with the hippocampus on the contralateral side. There was a mean $43.0 \pm 18.7\%$ increase in CA1 and a mean $86.6 \pm 27.5\%$ increase in CA3. Comparatively, the normoxic group did not display the same magnitude of increased staining on the injured side. There was a mean $22.6 \pm 8.2\%$ decrease in CA1 and a mean $21.5 \pm 33.3\%$ increase in CA3. There was no consistent pattern between the ipsilateral and contralateral hippocampal regions in either of the sham-operated groups. There was no significant difference in staining in the contralateral hippocampus among all four groups.

NeuN Analysis

For delayed immunohistochemical analysis, brain slices through the hippocampus were obtained from rats 1 week after exposure to one of three different conditions: injury with hyperoxic resuscitation ($n = 4$), injury with

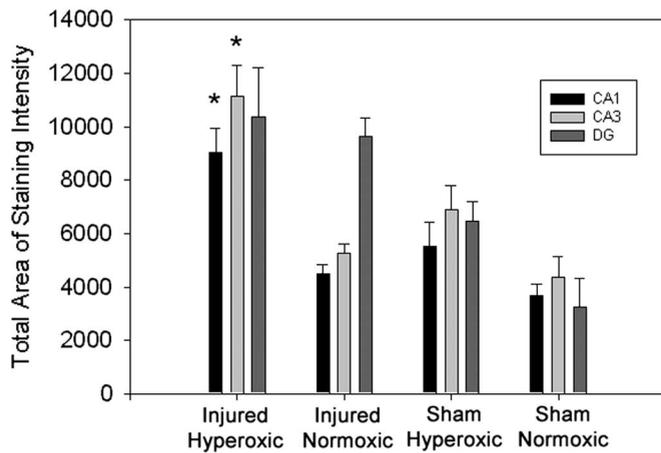


FIGURE 45.2 Quantification of 3-NT (1:500) staining 24 hours after CCI in regions of the hippocampus underlying the injured cortex. Staining area is quantified for each high-power field (40×) in square micrometers. In the CA1 and CA3 regions of the hippocampus of the injured hyperoxic group, there is a significant increase in 3-NT staining when compared with equivalent regions in the injured normoxic group and both sham-operated groups ($P < 0.05$). * $P < 0.05$ in equivalent regions of the other groups.

normoxic resuscitation (n = 4), and sham operation with hyperoxic resuscitation (n = 4). Slices were stained with the NeuN antibody (1:120,000). A sample photomicrograph from an injured hyperoxic rat is shown in *Figure 45.3A*. CA1 and CA3 regions of the hippocampus both ipsilateral and contralateral to the injury were analyzed under high-power magnification at 40×. There were two types of neurons counted: normal and abnormal. Normal neurons had the expected pyramidal shape with round nuclei. Abnormal neurons possessed vacuolated, pyknotic, or disrupted nuclei that were likely damaged cells in the process of dying (*Fig. 45.3B*).

Neuronal counts in the CA1 and CA3 regions of the hippocampus ipsilateral to injury are displayed in *Figure 45.4*. There was a significant reduction in CA1 neurons in both hyperoxic and normoxic injured rats when compared with the sham-operated rats ($P < 0.05$). However, there was no significant difference between the two injured groups. In CA3, there was no significant difference in neuronal counts between all groups.

The ratios between hippocampal neuronal counts on the injured side versus the noninjured side were determined to assess neuronal loss from injury (*Fig. 45.5*). In CA1, there was a mean cell loss of 22.3% in the hyperoxic group and 26.4% in the normoxic group. In CA3, the cell loss was less appreciable, with a mean of 9.9% in the hyperoxic group and 4.6% in the normoxic group. However, there was no significant difference between the hyperoxic and normoxic groups in CA1 or CA3.

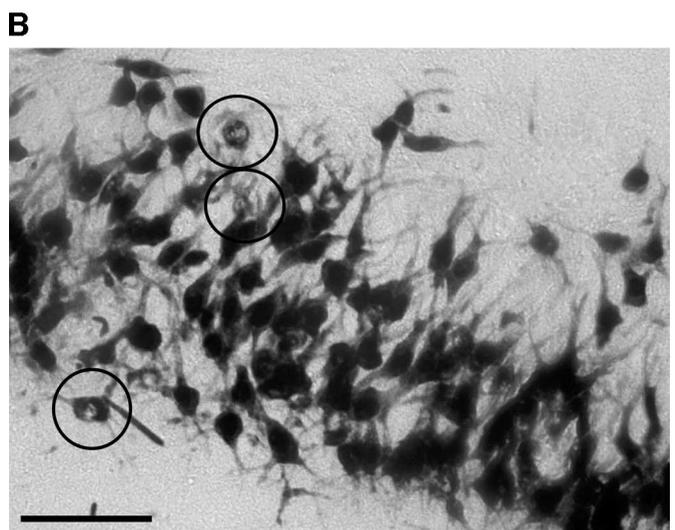


FIGURE 45.3 Sample photomicrographs of the hippocampus of an injured rat with NeuN staining (1:120,000) 7 days after CCI. *A*, A low-power view of the hippocampus is shown. *B*, a 40× magnification of the CA3 region is shown. Black circles indicate abnormal neurons with vacuolated nuclei, which likely represent neurons in the process of cell death. Scale bar = 50 μm.

Abnormal cell counts in the CA1 region of the hippocampus ipsilateral to the injury revealed $10.1 \pm 1.4\%$ abnormal cells in the hyperoxic group and $10.8 \pm 1.1\%$ in the normoxic group. In CA3, there were $8.1 \pm 1.1\%$ abnormal cells in the hyperoxic group and $12.4 \pm 2.1\%$ in the normoxic group. In both CA1 and CA3 regions, there was no significant difference in abnormal cell counts between the hyperoxic and normoxic groups.

DISCUSSION

Traumatic Brain Injury and Metabolic Dysfunction

Prospectively collected data from the Traumatic Coma Data Bank demonstrated that early hypoxemia in the field

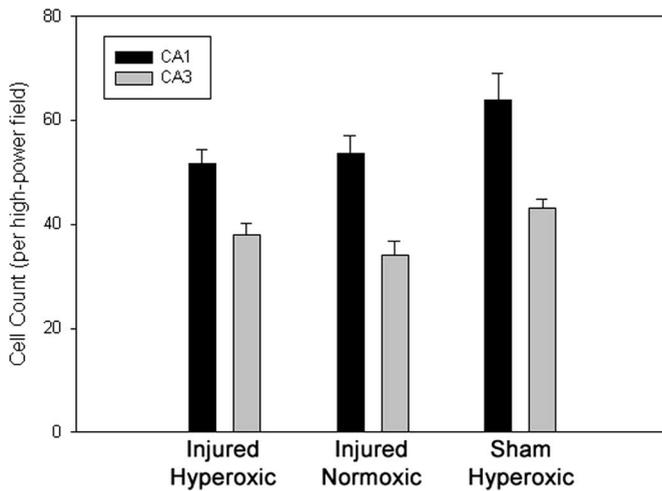


FIGURE 45.4 Neuronal counts per high-power field in the CA1 and CA3 regions of the hippocampus ipsilateral to injured cortex with NeuN staining 7 days after CCI. There is a significant reduction of neurons in the CA1 region of the hippocampus in both injured groups compared with the sham-operated group ($P < 0.05$). However, there is no significant difference between the hyperoxic- and normoxic-injured groups. In CA3, there is no significant difference in cell counts among groups.

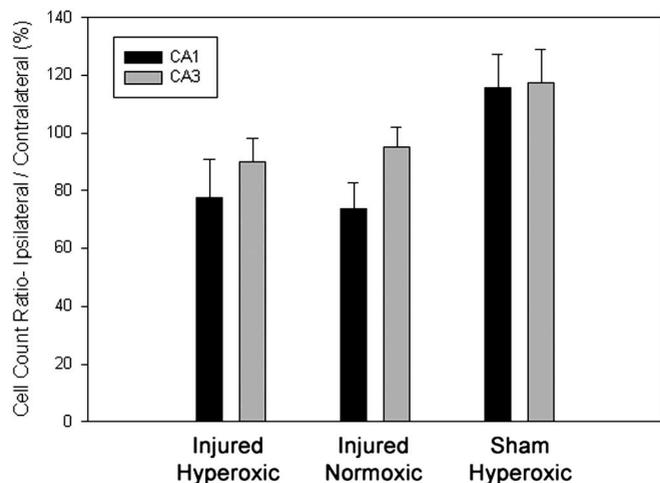


FIGURE 45.5 Ratio of neuronal counts of the ipsilateral versus contralateral hippocampus with NeuN staining 7 days after CCI. There is cell loss in the CA1 and CA3 regions of the injured hippocampus compared with the contralateral side in both injured groups. However, there is no significant difference in ratios between groups.

after severe TBI with $P_{aO_2} < 60$ mm Hg is associated with an increased morbidity and mortality.⁷ Therefore, recommendations from the Brain Trauma Foundation are to oxygenate to keep O_2 saturation $> 90\%$ or $P_{aO_2} > 60$ mm Hg to prevent hypoxia and secondary brain injury.^{6,15} In practice, ventilatory resuscitation with $F_{iO_2} 100\%$ is routinely used after

severe traumatic brain injury both during the prehospital management and during initial resuscitation while in the trauma center.

Recently, there have been clinical^{30,34,44} and laboratory³⁵ investigations that support the use of normobaric hyperoxia as a therapeutic measure in the initial treatment of severe TBI. This thought emanates from the fact that there is an increased metabolic demand of brain tissue after TBI, during which there is a shift from aerobic to anaerobic metabolism, as suggested by an increase in brain tissue lactate.^{5,48} The aim of hyperoxic ventilatory resuscitation is to restore aerobic metabolism and to ameliorate the mismatch between CBF and metabolic stimulation.

In an experimental model of TBI, animals resuscitated with an $F_{iO_2} 100\%$ had significantly decreased brain lactate levels compared with animals resuscitated with $F_{iO_2} 21\%$ as measured by microdialysis.³⁵ In a clinical study, Menzel et al.³⁰ measured brain tissue lactate and glucose levels in patients who received $F_{iO_2} 100\%$ for 6 hours after severe TBI. Brain tissue lactate levels decreased by 40% from baseline during this hyperoxic period. Tolia et al.⁴⁴ confirmed these results in their multicenter study where patients with severe TBI were given $F_{iO_2} 100\%$ for 24 hours, which resulted in a significant decrease in lactate and glutamate levels, as measured by microdialysis, when compared with a historical control group. Comparatively, in another clinical study of severe TBI using microdialysis, Magnoni et al.²⁸ found reduced levels of lactate with ventilation with $F_{iO_2} 100\%$ and reduced levels of pyruvate whereby the lactate/pyruvate ratio did not change. Therefore, they concluded that hyperoxia did not change the redox status of injured cells, and thus, there was no improvement in glucose metabolism. However, in the study by Tolia et al.,⁴⁴ there was a significant decrease in the lactate/pyruvate ratio with hyperoxia. The difference in the two studies is suggested to be attributable to the delayed initiation of hyperoxia in the Magnoni et al.²⁸ study.

Furthermore, there have been reports about the benefit of using hyperbaric oxygen therapy in an effort to increase brain tissue P_{O_2} after severe TBI.^{11,36,43} Similar to the use of normobaric hyperoxia, the therapeutic aim is to improve oxidative metabolism; however, with the use of these enhanced concentrations of oxygen, there must be further concern about oxidative damage to the injured cells.

Stroke and Cardiac Arrest

Previous studies on the effect of hyperoxia on ischemic brain injury from stroke are precursors to the experiments described in this article. Hyperoxia has been shown to be beneficial in experimental models of focal cerebral ischemia.^{13,40,41} One hundred percent O_2 is routinely administered to patients after an acute stroke, as a recommended intervention from the American Heart Association.¹ However, in a measure of patient survival after acute stroke of

various severities, Rønning et al.³⁷ demonstrated that supplemental 100% O₂ administered to patients with minor to moderate strokes for 24 hours reduced survival when compared with those who did not receive supplemental oxygen. In a canine model of cardiac arrest, dogs resuscitated with 21% O₂ demonstrated lower levels of oxidized brain lipids and improved neurological outcome compared with those resuscitated with 100% O₂.²⁵ These studies, in addition to other animal studies of global cerebral ischemia,^{32,49} caution the empiric usage of 100% O₂ after an acute stroke.

Previously, in our laboratory, the hypothesis that resuscitation with 100% O₂ after global cerebral ischemia results in increased ROS-induced damage was tested with the canine cardiac arrest model. 3-NT has been established as a marker for oxidative damage to proteins after peroxynitrite-mediated nitration of tyrosine.^{3,4,20,21} When the hippocampus was stained with the 3-NT antibody 2 hours after cardiac arrest, those animals resuscitated with 100% O₂ showed a significant increase in staining in all regions of the hippocampus compared with those resuscitated with normoxia.⁴⁶ Understanding that this model of global cerebral ischemia has many differences with models of TBI, our experiments were designed to address the possibility that exacerbation of ROS-induced protein damage in the hippocampus with hyperoxia could also be present after TBI.

Oxidative Stress

This study is consistent with previous studies in which there is increased production of ROS after experimental TBI.^{2,12,16–18,24,29,33} ROS, such as peroxynitrite, exhibit toxicity to the brain by way of their modification of macromolecules, especially DNA, and the induction of apoptotic and necrotic cell death pathways.²⁷ Investigators have described the time course of peroxynitrite-mediated oxidative damage to proteins using the 3-NT antibody in experimental models of TBI.^{18,31} Hall et al.¹⁸ showed that the highest intensity of staining was within the first 48 hours after injury in the neuronal perikarya and processes and microvessels. Our findings of significant 3-NT immunostaining 24 hours after CCI are consistent with the findings of Hall et al.¹⁸ Those rats in our study with hyperoxic resuscitation had an increased staining intensity on the injured side compared with the noninjured side of 43.0% in CA1 and 86.6% in CA3. However, this increased staining on the injured side was not as pronounced in the normoxic group. Moreover, the total amount of staining on the injured side in the normoxic group was no different than in the sham-operated groups. In contrast, there was a significantly increased amount of staining in the injured hippocampus of the hyperoxic group. Our findings suggest that normoxic ventilatory resuscitation provides significant protection against oxidative damage to proteins as measured at 24 hours after TBI when compared with hyperoxic ventilatory resuscitation.

In addition to the differences seen among the injured rats, there was also a noticeable difference between the sham-operated groups. Although not a statistically significant difference, the hyperoxic sham-operated rats had increased levels of 3-NT staining in the hippocampus when compared with the normoxic sham-operated rats, which suggests that hyperoxic ventilation alone may increase levels of oxidative stress. Similarly, in the study of ROS production from subdural hematoma induction, Doppenberg et al.¹² found increased levels of hydroxyl radical degradation products in noninjured animals after increasing the FiO₂ to 100%. These results support the hypothesis that in the presence of high oxygen tension, even under normobaric conditions and in the noninjured brain, there is increased ROS production. These findings in sham-operated animals warrant additional caution with respect to the administration of large concentrations of ventilatory oxygen.

Delayed neuronal counts at 1 week after injury were consistent with previous studies using the CCI model. The CA1 and CA3 regions of the hippocampus showed appreciable cell loss on the injured side. In our analysis, the CA1 region was affected more than the CA3 region. These results differ from previous studies using the CCI model where the CA3 region was the more affected population of neurons.^{8,14,42,45} The difference may be due to the parameters of the impact: depth of injury and impact velocity. Our CCI parameters allowed for a more shallow depth and a higher impact velocity, which may have resulted in an increased susceptibility of CA1 neurons to cell death. The appearance of neurons with pyknotic or vacuolated nuclei in the hippocampus on the side of injury is also consistent with past studies using the CCI model.^{9,10} These neurons, which have been found to be present up to 2 weeks after injury, are likely destined to die by either necrosis or apoptosis.

Studies have supported the use of normobaric hyperoxia in the initial treatment after TBI because of the favorable shift from anaerobic to aerobic metabolism.^{30,34,35,44} However, our analysis of neuronal cell loss and abnormal cell counts did not show any beneficial effect of hyperoxic resuscitation. At 1 week after injury, rats that were ventilated with FiO₂ 100% for 1 hour after CCI showed no significant difference in neuronal loss and abnormal cell morphology than those that were ventilated with room air. Perhaps a lengthier period of hyperoxic ventilation after CCI may have been required to establish a more favorable environment for aerobic metabolism in the injured tissue; however, as demonstrated by our findings with 3-NT staining, one would also expect a significantly heightened level of oxidative damage with extended hyperoxic ventilation. It is possible that as a consequence of hyperoxia, there are at least two major processes, a shift toward aerobic metabolism and oxidative damage, both of which may affect neuronal survival; there-

fore, further investigation is required to define their respective roles in delayed neuronal function and survival after TBI.

Clinical Implications

With early hypoxemia as a reliable predictor for morbidity and mortality after severe TBI, endotracheal intubation and supplemental oxygenation is indeed a crucial intervention in the early resuscitation of a patient after severe TBI. However, when ventilation with FiO_2 100% becomes prolonged, this administration of normobaric hyperoxia then moves beyond an early intervention to become a deliberate treatment. As with any treatment, a dose-response curve and consideration for treatment toxicity is essential. In light of the findings by Magnoni et al.²⁸ that demonstrate a lack of improvement in cerebral metabolism with hyperoxic resuscitation, the benefit of normobaric hyperoxia after severe TBI is at least debatable. Moreover, our finding of a significant increase in oxidative damage to proteins with just 1 hour of hyperoxic ventilation in an experimental TBI model demonstrates the potential toxicity of the purported treatment with normobaric hyperoxia. This concern for ROS-mediated damage to the brain with hyperoxia compounds what has already been established about oxygen toxicity to the lungs.

In the prehospital setting, ventilatory resuscitation with 100% O_2 may be the most effective method of avoiding early hypoxemia after severe TBI. However, if efforts toward ameliorating ROS-mediated damage are to be made, this large concentration of oxygen can rapidly and easily be adjusted. Because pulse oximetry and arterial blood gas analyses are routinely monitored for early hypoxemia, the same modalities could also be used to monitor for the potential toxicity of hyperoxia. These modalities could be monitored with a predetermined set of parameters to avoid the harmful effects of either extreme in oxygenation. Similar parameters could also be applied to brain tissue oximetry. Nevertheless, further investigation into hyperoxic ventilation after severe TBI is required to establish how its potential metabolic benefit and its potential for oxidative damage impact neuronal survival and neurological outcome.

CONCLUSION

Ventilatory resuscitation with 100% O_2 is routinely administered after severe TBI to prevent early hypoxemia. In addition, the use of normobaric hyperoxia after severe TBI has recently been supported for the main therapeutic goal of shifting from anaerobic metabolism to aerobic metabolism in the injured brain. Other investigators have called into question this metabolic benefit. Furthermore, the use of normobaric hyperoxia heightens the concern for oxygen toxicity and ROS-mediated damage to the brain. Our results show that rats administered 100% O_2 for 1 hour after CCI had significantly increased levels of ROS-mediated damage to proteins in the CA1 and CA3 regions of the hippocampus when compared

with rats administered room air. Levels of ROS-mediated protein nitration in the normoxic group were no different than those of noninjured rats. Additionally, there was no beneficial effect of hyperoxic resuscitation on neuronal survival or abnormal neuronal morphology 1 week after injury. These results warrant additional caution in the empiric use of hyperoxic resuscitation in the treatment of severe TBI. If future studies support this concern for increased ROS-mediated damage with hyperoxia, then efforts will need to be made toward early adjustments in the O_2 concentration administered during resuscitation after severe TBI.

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A—AU: Reference number 39 was skipped; thus, references have been renumbered
