

Normoxic ventilatory resuscitation following controlled cortical impact reduces peroxynitrite-mediated protein nitration in the hippocampus

Laboratory investigation

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Object. Ventilatory resuscitation with 100% O₂ after severe traumatic brain injury (TBI) raises concerns about the increased production of reactive oxygen species (ROS). The product of peroxynitrite-mediated tyrosine residue nitration, 3-nitrotyrosine (3-NT), is a marker for oxidative damage to proteins. The authors hypothesized that posttraumatic resuscitation with hyperoxia (100% fraction of inspired oxygen [FiO₂] concentration) results in increased ROS-induced damage to proteins compared with resuscitation using normoxia (21% FiO₂ concentration).

Methods. Male Sprague–Dawley rats underwent controlled cortical impact (CCI) injury and resuscitation with either normoxic or hyperoxic ventilation for 1 hour (5 rats per group). Twenty-four hours after injury, rat hippocampi were evaluated using 3-NT immunostaining. In a second experiment, animals similarly underwent CCI injury and normoxic or hyperoxic ventilation for 1 hour (4 rats per group). One week after injury, neuronal counts were performed after neuronal nuclei immunostaining.

Results. The 3-NT staining was significantly increased in the hippocampi of the hyperoxic group. The normoxic group showed a 51.0% reduction of staining in the CA1 region compared with the hyperoxic group and a 50.8% reduction in the CA3 region ($p < 0.05$, both regions). There was no significant difference in staining between the injured normoxic group and sham-operated control groups. In the delayed analysis of neuronal survival (neuronal counts), there was no significant difference between the hyperoxic and normoxic groups.

Conclusions. In this clinically relevant model of TBI, normoxic resuscitation significantly reduced oxidative damage to proteins compared with hyperoxic resuscitation. Neuronal counts showed no benefit from hyperoxic resuscitation. These findings indicate that hyperoxic ventilation in the early stages after severe TBI may exacerbate oxidative damage to proteins. (DOI: 10.3171/JNS/2008/108/01/0124)

KEY WORDS • controlled cortical impact • free radical • hyperoxia • nitrotyrosine • reactive oxygen species • traumatic brain injury

IMPROVEMENT of cerebral oxygenation after human TBI is one of the main therapeutic goals when attempting to prevent secondary brain injury. Cerebral edema—and often decreased cerebral blood flow—occurs after severe TBI, 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 FiO₂ (100% concentration).³¹ In the prehospital setting, endotracheal intubation and initial resuscitation with an FiO₂ concentration of 100% is routinely used in patients after severe TBI. In an effort to prevent hypoxia and secondary brain injury, it has been recommended as a guideline to avoid O₂ saturation level < 90% in the field or a PaO₂ level < 60 mm Hg.⁶ Often, however, high concentrations of O₂ are admin-

istered continuously during resuscitation and throughout the first 24 hours after injury, which results in PaO₂ levels well above physiological conditions.

Despite the seemingly beneficial effect of increased FiO₂ concentration (or normobaric hyperoxia) on cerebral metabolism after TBI,⁴⁵ 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 high concentrations of O₂. 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.^{22,40} The question also remains as to how much O₂, when delivered at high concentrations to the lungs and blood stream, is actually effectively delivered to the brain tissue.^{23,28,39}

The aim of this study is to determine the effect of hyperoxic (100% FiO₂ concentration) versus normoxic (21% FiO₂ concentration) ventilatory resuscitation after TBI on the production of ROS by using an experimental model of CCI injury. Peroxynitrite is formed from a reaction be-

Abbreviations used in this paper: CCI = controlled cortical impact; DAB = 3,3'-diaminobenzidine; DG = dentate gyrus; FiO₂ = fraction of inspired O₂; HPF = high-power field; KPBS = potassium phosphate-buffered saline; NeuN = neuronal nuclei; ROS = reactive oxygen species; SEM = standard error of the mean; TBI = traumatic brain injury; 3-NT = 3-nitrotyrosine.

Reduced oxidative damage with normoxic ventilation after trauma

tween the ROS NO and superoxide. The product of peroxynitrite-mediated nitration of tyrosine residues, 3-NT, 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 injury.

Materials and Methods

Animal Population

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

Surgical Procedures

Animals were placed in a plexiglass chamber and anesthesia was induced by inhalation of 4.5% isoflurane in pressurized room air for 2–3 minutes. The animals were then endotracheally intubated with a 14-gauge angiocath and placed on positive pressure ventilation using a small animal ventilator (Harvard Apparatus). Maintenance anesthesia was delivered using 3% isoflurane. Each animal was placed in a stereotactic frame. A rectal temperature probe and a heating blanket (Henry Schein, Inc.) were used to maintain body temperature at 37°C. The mean temperature at the time of CCI was $36.9 \pm 0.1^\circ\text{C}$ (range 34.4–37.7°C).

After a midline scalp incision, the left parietal bone was exposed. A left parietal craniotomy was performed with the aide of a surgical microscope and a dental drill (Fine Science Tools). Next, the CCI device was calibrated with respect to the exposed dura mater within the craniotomy. The parameters of impact in injured animals were a depth of 1.5 mm, a mean velocity of 5.62 ± 0.04 m/second (range 5.24–5.88 m/second), and a duration of 50 msec. After impact, the animals in the hyperoxic group were ventilated with 100% O₂ for 1 hour. Animals in the normoxic group continued to be ventilated with room air (21% FiO₂ concentration) for 1 hour. The craniotomy site was covered with dental acrylic and the scalp incision was closed with silk sutures. Sham-operated groups received a craniotomy without CCI injury and were ventilated with either hyperoxia or normoxia for 1 hour. Arterial blood gas analysis was performed in all animals 30 minutes after injury using blood obtained from the tail artery. After 1 hour of resuscitation, the isoflurane was discontinued. The animals were extubated after exhibiting spontaneous respiration and movement.

Immunohistochemical Findings

3-Nitrotyrosine Staining. Twenty-four hours after injury, animals were injected with a lethal dose of intraperitoneal sodium pentobarbital. Animals were perfused first with intracardiac saline and then with 4% paraformaldehyde. The brains were removed and postfixed in a 2.5% acrolein (EM grade, Polysciences, Inc.) solution in 4% paraformaldehyde, at pH 6.8, for 1 hour, and then stored in 4% paraformaldehyde for 1 day. The brains were sliced to isolate 3-mm coronal blocks that included the hippocampi. These blocks were 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 prior to staining.⁴⁸ Sections were then washed in 0.1M Tris buffer solution 6 times at pH 7.6, for 10 minutes each time, to rinse out the cryoprotectant. They were 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 Lorich and colleagues.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, and washed in tap water for 10 minutes. Slides were then submerged in a boiling citrate solution for 10

minutes and allowed to soak in the hot solution for another 10 minutes. They were washed in distilled H₂O for 5 minutes and Tris buffer 3 times, 5 minutes each time. The 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-NT antibody (1:500, Upstate) overnight at 4°C. The slices were washed in Tris buffer 2 times, for 5 minutes each time, 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 2 times, for 5 minutes each time, slices were stained with Ni-DAB chromogen using the VECTASTAIN Elite ABC kit (Vector Laboratories). Stained slices were analyzed for intensity of 3-NT staining. Quantitative analyses were performed using a computer-assisted image analysis system, which consisted of a Nikon Eclipse 800 photomicroscope, a Retiga 1300 cooled CCD digital camera (Biovision Technologies), and a Macintosh G4 computer with IP Spectrum software (Scientific Image Processing, version 3.9.3, Scanalytics). The total area of staining was determined in regions of the hippocampus using a magnification of 40. For each subject, an equivalent slice through the hippocampus was analyzed in the CA1, CA 2/3, and DG regions in both sides ipsilateral and contralateral to the injury. For each region of the hippocampus, 3 fields were analyzed using a magnification of 40. 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, to account for variability in general staining intensity of the individual slices, segmentation values were adjusted to eliminate any background staining, after which the total area of staining (in μm^2) was determined. The resultant value represented a measure of the difference in gray level between that of the staining within the cells and that of the neuropil in the background. All slices were analyzed by the same examiner (E.S.A.) for consistency.

Neuronal Nuclei Staining. For delayed analysis of neuronal survival (7 days after injury), animals were perfused and brain slices were prepared following the same method described earlier 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 described previously by Hoffman and colleagues.¹⁹ Briefly, sections were washed in 0.05 M KPBS 6 times, for 10 minutes each time, to rinse out the cryoprotectant. The sections were then incubated in a sodium borohydride solution as described earlier and rinsed multiple times in KPBS. The slices were then incubated with the primary antibody, mouse monoclonal anti-NeuN (Chemicon, 1:120,000), in KPBS with 0.4% Triton-X for 1 hour at room temperature, then for 24 hours at 4°C. Sections were then rinsed again in KPBS 6 times, for 10 minutes each time. 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 5 times, for 10 minutes each time, sections were prepared using the VECTASTAIN Elite ABC kit. Sections were then rinsed in KPBS 3 times, for 5 minutes each time; in 0.175M sodium acetate 3 times, for 5 minutes each time; and in KPBS 3 times, for 5 minutes each time. The sections were placed into a Ni-DAB H₂O₂ chromogen solution (250 mg Ni sulfate, 2 mg DAB, and 8.3 μl 3% H₂O₂ /10 ml 0.175 sodium acetate solution). Sections were left in solution for 10 minutes and staining was terminated by transferring sections to the sodium acetate solution. The stained sections were mounted on glass slides, dehydrated, and coverslipped with Histomount (Zymed Laboratories). For neuronal quantification, the same computer-assisted image analysis system described previously for 3-NT was used. For each animal, 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, 2 HPFs at a magnification of 40 were analyzed. The number of normal neurons was determined. In addition, abnormal neurons that exhibited fragmented, pyknotic, or absent nuclei were also counted. All slices were analyzed by the same examiner (C.L.R.) for consistency. The examiner was blinded to the ventilatory treatment (normoxic or hyperoxic) but not to whether the animal was sham-operated or injured.

Statistical Analysis

One-way analysis of variance (Student-Newman-Keuls method) was used for data analysis. All comparisons with a probability value < 0.05 were considered statistically significant. All values are expressed as means \pm SEMs.

Results

Arterial Blood Gas Analysis

For the animals analyzed for 3-NT, mean PO_2 , PCO_2 , and pH values obtained from tail arterial blood after 30 minutes of ventilatory resuscitation following CCI injury are shown in Table 1. In the 3-NT immunohistochemistry experiment, mean PO_2 values for the hyperoxic ventilated animals were 396.5 ± 26.9 mm Hg for the injured animals and 346.0 ± 30.5 mm Hg for the sham-operated animals. These values were significantly elevated when compared with their normoxic group counterparts, with PO_2 values of 105.6 ± 4.3 mm Hg and 102.8 ± 8.7 mm Hg, respectively ($p < 0.001$). Arterial blood gas analysis for animals analyzed for NeuN are shown in Table 2. Of the rats that received hyperoxic resuscitation, injured animals had PO_2 values of 306.8 ± 25.6 mm Hg and sham-operated animals 346.7 ± 28.7 mm Hg; similar to the 3-NT analysis results, these values were significantly elevated compared with the mean PO_2 values from normoxic animals of 128.1 ± 16.8 mm Hg ($p < 0.001$). In both experiments, there was no significant difference in PCO_2 or pH values between groups.

Analysis of 3-NT

In the 3-NT experiment, animals were subjected to 1 of 4 conditions as follows: CCI injury with hyperoxic resuscitation (5 rats), CCI injury with normoxic resuscitation (5 rats), sham operation with hyperoxic resuscitation (5 rats), or sham operation with normoxic resuscitation (4 rats). To determine levels of oxidative stress, brain slices through the hippocampus were obtained 24 hours after CCI injury and stained with the 3-NT antibody (Fig. 1). Quantification of 3-NT staining in the hippocampus ipsilateral to the injured cortex is displayed in Fig. 2. In the hippocampal region of CA1, animals resuscitated with normoxia showed a 51.0% reduction in staining when compared with those animals resuscitated with hyperoxia ($p < 0.05$). In the CA3 region, normoxic animals showed a 50.8% reduction in staining when compared with hyperoxic animals ($p < 0.05$). 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 significant difference in staining in these regions between the injured normoxic group and both sham-operated groups. There was also no significant difference in the staining of the DG among all groups. Of note, the sham-operated hyperoxic group showed increased staining in all hippocampal regions compared with the sham-operated normoxic group, but these differences were not statistically significant ($p = 0.679$ in CA1, $p = 0.475$ in CA3, and $p = 0.271$ 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 staining increase of $43.0 \pm 18.7\%$ in the

TABLE 1

Arterial blood gas analysis results during animal ventilatory resuscitation after CCI in the 3-NT immunohistochemistry experiment*

Group (no. of rats)	PO_2 (mm Hg)	PCO_2 (mm Hg)	pH
injured hyperoxic (5)	$396.5 \pm 26.9^\dagger$	32.0 ± 3.6	7.51 ± 0.04
injured normoxic (5)	105.6 ± 4.3	31.4 ± 6.1	7.46 ± 0.06
sham hyperoxic (5)	$346.0 \pm 30.5^\dagger$	30.6 ± 2.6	7.52 ± 0.03
sham normoxic (4)	102.8 ± 8.7	33.4 ± 2.6	7.49 ± 0.03

*Values are expressed as means \pm SEMs.

$^\dagger p < 0.001$ compared with normoxic group counterparts.

CA1 region and an $86.6 \pm 27.5\%$ increase in the CA3 region. 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 4 groups.

Neuronal Nuclei Analysis

For delayed immunohistochemical analysis, brain slices through the hippocampus were obtained from animals 1 week after exposure to 1 of 3 different conditions: CCI injury with hyperoxic resuscitation (4 rats), CCI injury with normoxic resuscitation (4 rats), or sham operation with hyperoxic resuscitation (4 rats). Slices were stained using the NeuN antibody (1:120,000). A sample photomicrograph from an injured hyperoxic animal is shown in Fig. 3 left. The CA1 and CA3 regions of the hippocampus both ipsilateral and contralateral to the injury were analyzed under high-power magnification ($\times 40$). Both normal and abnormal neurons were counted. 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. 3 right).

Neuronal counts in the CA1 and CA3 regions of the hippocampus ipsilateral to the injury site are displayed in Fig. 4. There was a significant reduction in CA1 neurons in both hyperoxic and normoxic injured animals when compared with the sham-operated animals ($p < 0.05$). There was no significant difference, however, between the 2 injured groups. In the CA3 region, there was no significant difference in neuronal counts among any of the groups.

TABLE 2

Arterial blood gas analysis results during animal ventilatory resuscitation after CCI in the NeuN experiment*

Group (no. of rats)	PO_2 (mm Hg)	PCO_2 (mm Hg)	pH
injured hyperoxic (4)	$306.8 \pm 25.6^\dagger$	35.4 ± 1.9	7.48 ± 0.02
injured normoxic (4)	128.1 ± 16.8	32.7 ± 4.3	7.50 ± 0.04
sham hyperoxic (4)	$346.7 \pm 28.7^\dagger$	34.2 ± 2.6	7.52 ± 0.03

* Values are expressed as means \pm SEMs.

$^\dagger p < 0.001$ compared with normoxic group counterparts.

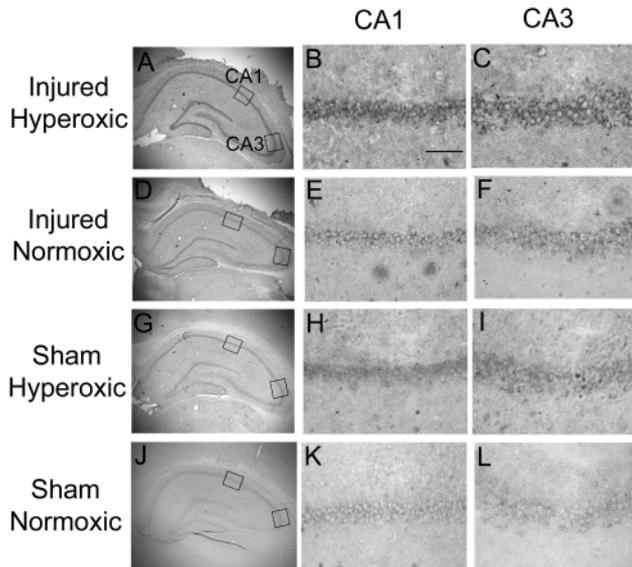


FIG. 1. Sample photomicrographs of the hippocampus underlying the injured cortex with 3-NT (1:500) staining 24 hours after CCI injury for each of the subject groups as follows: injured hyperoxic (A–C), injured normoxic (D–F), sham hyperoxic (G–I), and sham normoxic (J–L). Photomicrographs in the first column (A, D, G, and J) are low-power views ($\times 2$) of the hippocampus. The damaged cortex can be seen in both injured animals (A and D). Box outlines indicate the CA1 and CA3 regions of the hippocampus, which are shown to the right at a magnification of 40 (CA1: B, E, H, and K; CA3: C, F, I, and L). The injured hyperoxic groups 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. Bar = 50 μm .

The ratios between hippocampal neuronal counts on the injured side versus the noninjured side were determined to assess neuronal loss from injury (Fig. 5). In the CA1 region, there was a mean cell loss of 22.3% in the hyperoxic group and 26.4% in the normoxic group. In the CA3 region, the cell loss was less appreciable with a mean of 9.9% in the hyperoxic group and 4.6% in the normoxic group. There was no significant difference, however, between the hyperoxic and normoxic groups in either the CA1 or CA3 regions.

Abnormal cell counts in the CA1 region of the hippocampus ipsilateral to the injury revealed a total of $10.1 \pm 1.4\%$ abnormal cells in the hyperoxic group and $10.8 \pm 1.1\%$ in the normoxic group. In the CA3 region, 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 the CA1 and CA3 regions, there were no statistically significant differences 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 after severe TBI with $\text{PaO}_2 < 60$ mm Hg is associated with increased morbidity and mortality rates.⁷ Therefore, recom-

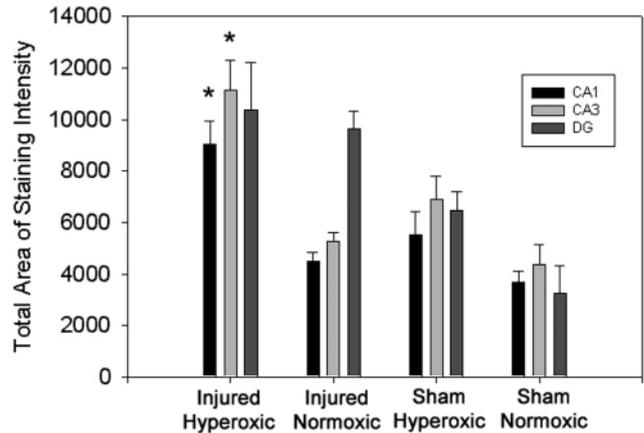


FIG. 2. Bar graph showing quantification of 3-NT (1:500) staining 24 hours after CCI injury in regions of the hippocampus underlying the injured cortex. Staining area is quantified for each HPF ($\times 40$) in μm^2 . In the CA1 and CA3 regions of the hippocampus of the injured hyperoxic group, there is a significant increase in 3-NT staining when compared with equivalent regions in the injured normoxic group and both sham-operated groups. * $p < 0.05$ in equivalent regions of the other groups.

mendations from the Brain Trauma Foundation are to oxygenate patients to keep O_2 saturation $> 90\%$ or $\text{PaO}_2 > 60$ mm Hg to prevent hypoxia and secondary brain injury.^{6,15} In practice, ventilatory resuscitation with 100% FiO_2 concentration is routinely used after severe TBI both during the prehospital management and during initial resuscitation while in the trauma center.

Recently, there have been clinical^{31,35,47} and laboratory³⁶ investigations that support the use of normobaric hyperoxia as a therapeutic measure in the initial treatment of severe TBI. This belief 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 a rise in brain tissue lactate.^{5,49} The aim of hyperoxic ventilatory resuscitation is to restore aerobic metabolism and to ameliorate the mismatch between cerebral blood flow and metabolic stimulation.

In an experimental model of TBI,³⁶ animals resuscitated with 100% FiO_2 showed significantly decreased brain lactate levels compared with animals resuscitated with 21% FiO_2 as measured using microdialysis. In a clinical study, Menzel and colleagues³¹ measured brain tissue lactate and glucose levels in patients who received 100% FiO_2 for 6 hours after severe TBI; brain tissue lactate levels decreased by 40% from baseline during this hyperoxic period. Tolia and associates⁴⁵ confirmed these results in their multicenter study in which patients with severe TBI were given 100% FiO_2 for 24 hours, which resulted in a significant decrease in lactate and glutamate levels when compared with a historical control group, as measured using microdialysis. Comparatively, in another clinical study of severe TBI using microdialysis, Magnoni and colleagues²⁸ found reduced levels of lactate with ventilation using 100% FiO_2 , as well as reduced levels of pyruvate (the lactate/pyruvate ratio did not change). Therefore, Magnoni and colleagues concluded that hyperoxia did not change the redox status of injured cells, and thus, there was no improvement in glucose metabolism. In the study by Tolia and associates,⁴⁵ however,

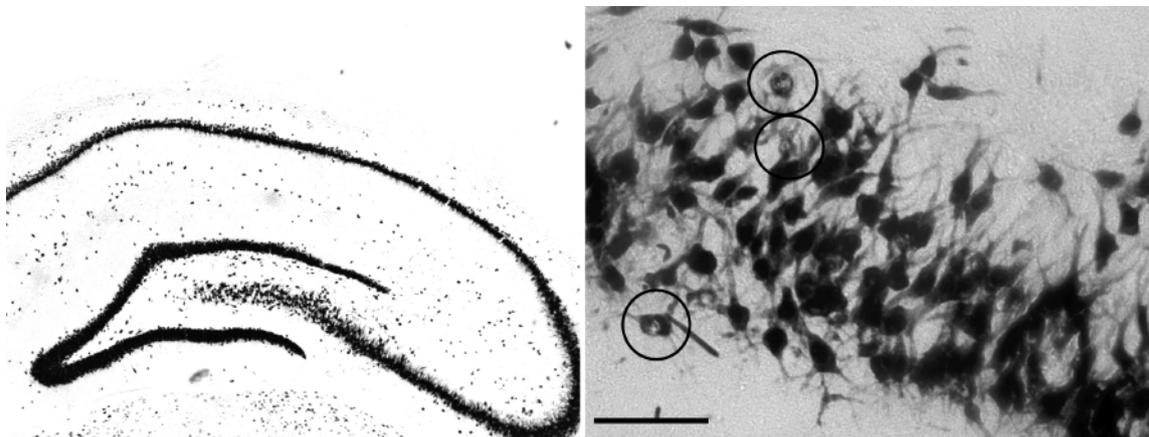


FIG. 3. Sample photomicrographs of the hippocampus of an injured animal with NeuN staining (1:120,000) 7 days after CCI injury. *Left:* A low-power view ($\times 2$) of the hippocampus is shown. *Right:* The CA3 region is shown at a magnification of 40. *Circles* indicate abnormal neurons with vacuolated nuclei, which likely represent neurons in the process of cell death. Bar = 50 μm .

there was a significant decrease in the lactate/pyruvate ratio with hyperoxia. The difference in the 2 studies is suggested to be attributable to the delayed initiation of hyperoxia in the study by Magnoni and coworkers.⁴⁵

Furthermore, there have been reports concerning the benefit of using hyperbaric O₂ therapy in an effort to increase brain tissue PO₂ after severe TBI.^{11,37,44} Similar to the use of normobaric hyperoxia, the therapeutic aim of hyperbaric O₂ therapy is to improve oxidative metabolism; however, with the use of these enhanced concentrations of O₂, there must be further concern about oxidative damage to the injured cells.

Stroke and Cardiac Arrest

Previous studies of the effect of hyperoxia on ischemic

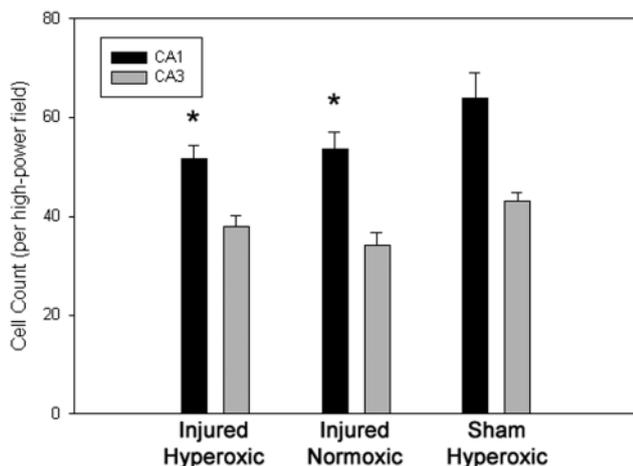


FIG. 4. Bar graph of the neuronal counts per HPF in the CA1 and CA3 regions of the hippocampus ipsilateral to the injured cortex with NeuN staining 7 days after CCI injury. A significant reduction of neurons in the CA1 region of the hippocampus in both injured groups was found compared with the sham-operated group (* $p < 0.05$). There is no significant difference between the hyperoxic and normoxic injured groups. In the CA3 region, there is no significant difference in cell counts between groups.

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.^{13,41,42} A 100% concentration of O₂ is routinely administered to patients after an acute stroke as a recommended intervention from the American Heart Association.¹ In a measure of patient survival after acute stroke of various severities, however, Rønning and Guldvog³⁸ 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 O₂. 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,^{33,50} caution against 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 using the canine cardiac arrest model. It has been established that 3-NT is 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.⁴⁷ Given the 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 showing increased production of ROS after experimental TBI.^{2,12,16-18,24,29,30,34} Reactive oxygen species, such as peroxynitrite, exhibit toxicity to the brain by modifying macromolecules, especially DNA, and by the induction of apoptotic and ne-

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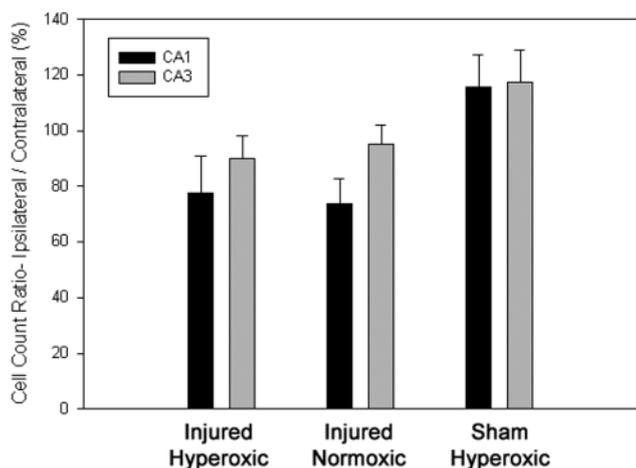


FIG. 5. Bar graph of the ratio of neuronal counts of the ipsilateral versus contralateral hippocampus with NeuN staining 7 days after CCI injury. There is cell loss in the CA1 and CA3 regions of the injured hippocampus compared with the contralateral side in both injured groups, but no significant difference in ratios between groups.

croic 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.^{18,32} Hall and associates¹⁸ showed that the highest intensity of staining was within the first 48 hours after injury in the neuronal perikarya and processes, and in microvessels. Our findings of significant 3-NT immunostaining 24 hours after CCI injury are consistent with the findings of Hall and colleagues, with the exception of the lack of microvessel staining in our injured animals. This particular difference, however, does not rule out oxidative damage to microvessels after TBI with hyperoxic ventilation. The 3-NT staining of microvessels may vary with the timing of analysis after injury, the experimental model of TBI, and the severity of injury.

Those animals in our study with hyperoxic resuscitation showed an increased staining intensity of 3-NT on the injured side compared with the noninjured side of 43.0% in the CA1 region and 86.6% in the CA3 region; 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.

Among the hippocampal regions, the CA1 and CA3 regions showed the most pronounced increase in 3-NT staining in the hyperoxic group. There was no difference between groups in the DG. Compared with other hippocampal regions, the DG was densely packed with neurons, which resulted in a high level of background staining. Any difference between groups would likely be less pronounced in view of this increased background staining.

In addition to the differences noted among the injured

animals, there was also a noticeable trend between the sham-operated groups. Although not a statistically significant difference, the hyperoxic sham-operated animals had elevated levels of 3-NT staining in the hippocampus when compared with the normoxic sham-operated animals, which suggests that hyperoxic ventilation alone may increase levels of oxidative stress. Similarly, in the study of ROS production from subdural hematoma induction, Doppenberg and colleagues¹² found increased levels of hydroxyl radical degradation products in noninjured animals after increasing the FiO_2 to 100%. These results support the hypothesis that in the presence of high O_2 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 high concentrations of ventilatory O_2 .

Delayed neuronal counts at 1 week after injury were consistent with results from past studies using the CCI injury 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 in which the CA3 region was the site of the more affected population of neurons.^{8,14,43,46} This difference may be due to the parameters of the impact, that is, the 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 for as long as 2 weeks after injury, are likely destined to die due to 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.^{31,35,36,45} Our analysis of neuronal cell loss and abnormal cell counts, however, did not show any beneficial effect of hyperoxic resuscitation. At 1 week after injury, animals that were ventilated with 100% FiO_2 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; but, 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 2 major processes, a shift toward aerobic metabolism and oxidative damage, both of which may affect neuronal survival; therefore, further investigation is needed to define their respective roles in delayed neuronal function and survival after TBI. In the future, behavioral outcome studies would be important to demonstrate the impact of hyperoxic ventilation on neurological function after TBI.

Clinical Implications

With early hypoxemia as a reliable predictor for morbidity and death after severe TBI, endotracheal intubation and supplemental oxygenation is indeed a crucial intervention in the early resuscitation of a patient after severe

TBI. When ventilation with 100% FiO₂ becomes prolonged, however, 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 view of the findings of Magnoni et al.²⁸ that demonstrate a lack of improvement in cerebral metabolism using 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 after 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 those concerns already established about O₂ toxicity to the lungs.

In the prehospital setting, ventilatory resuscitation with 100% O₂ may be the most effective method of avoiding early hypoxemia after severe TBI. Yet if efforts toward ameliorating ROS-mediated damage are to be made, this high concentration of O₂ can rapidly and easily be adjusted. Given that pulse oximetry and arterial blood gas analyses are routinely monitored for early hypoxemia, the same modalities could also be used to monitor 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 necessary to establish how its potential metabolic benefit and its potential for oxidative damage impact neuronal survival and neurological outcome.

Conclusions

Ventilatory resuscitation with 100% O₂ 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 questioned this metabolic benefit. Furthermore, the use of normobaric hyperoxia heightens the concern for O₂ toxicity and ROS-mediated damage to the brain. Our results show that animals administered 100% O₂ for 1 hour after CCI injury had significantly increased levels of ROS-mediated damage to proteins in the CA1 and CA3 regions of the hippocampus when compared with animals administered room air. Levels of ROS-mediated protein nitration in the normoxic group were no different than those of uninjured animals. 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 O₂ concentration administered during resuscitation after severe TBI.

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