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Emergency Interventions After Severe Traumatic Brain Injury in Rats: Effect on Neuropathology and Functional Outcome

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Traumatic brain injury (TBI) contributes to combat casualty morbidity and mortality. Our hypothesis is that optimal manipulation of practical interventions applicable in the emergency treatment of severe TBI (respiratory management, temperature control, and sedation) can reduce brain injury in a rat model of brain contusion, and thereby improve functional and neuropathological outcome. In the first year of funding, we addressed the first Technical Objective of our proposal - to perform a comprehensive study of the effects of mechanical ventilation strategies on outcome. We found that aggressive hyperventilation applied for 4 h immediately after injury is detrimental (vs normal ventilation), and increases neuronal death in vulnerable brain regions.

Also, to set the stage for the evaluation of therapies targeting improved outcome after TBI (proposed in Technical Objectives 2-4), the severity of the insult was increased in our model. This was done by more accurately simulating the field scenario (adding a secondary insult).

Finally, another injury station was established and a technician was trained to perform the studies proposed in y 2-3. Dr. Michael Forbes, a fellow completed his training during this year and was the first author of the manuscript described above.

Head Injury, Hyperventilation, Hypothermia, Trauma, Apoptosis, Excitotoxicity, Sedation, Oxygen

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(5) INTRODUCTION

In our application, we highlighted the fact that traumatic brain injury (TBI) is an important contributor to combat casualty morbidity and mortality. We also stated that although progress has been made in the development of rodent models of TBI (such as the controlled cortical impact (CCI) model), most of the studies with these models have focused on molecular mechanisms of damage. Because there has been a paucity of application of practical emergency interventions in TBI models, we felt that it was essential to address this deficiency and that this strategy could have important implications for field and emergency management of both soldiers and civilians with severe TBI. Our overall hypothesis is that optimal manipulation of practical interventions applicable in the emergency treatment of severe TBI (respiratory management, temperature control, and sedation) can reduce secondary brain injury in a rat model of brain contusion, and thereby improve functional and/or neuropathological outcome.

In the first year of funding, we addressed the most important aspect of the first Technical Objective of our proposal—namely—to perform a comprehensive study of the effects of mechanical ventilation strategies (as applied by the first responder in the field) on both functional and neuropathological outcome in our model. We found that aggressive, prophylactic hyperventilation (HV) applied for 4 h immediately after injury is detrimental (vs ventilation to a normal PaCO\textsubscript{2}, normal ventilation [NV]), and leads to an increase in the amount of neuronal death in selectively vulnerable brain regions. This study, is now in press as a full manuscript in the Journal of Neurosurgery (1), (also see Appendix #1). We were pleased that the reviewers indicated that this was an important study which would be cited often.

In addition, to set the stage for the evaluation of therapies targeting improvement in outcome after severe TBI (as proposed studies in technical objectives 2-4), it appeared that it would be optimal to increase the severity of the insult in our model. This was done by attempting to more accurately simulate the field scenario—i.e., adding a 30 min period of moderate hypoxemia to the insult. The characterization of that model for our future studies will also be described below.

During the first year of funding, Henry Alexander, an experienced technician assumed the technical duties of the injury model and has successfully learned the model to perform all of the subsequent injury studies in years 2 and 3. Also, a new injury device and station were purchased and is in operation for these studies. Finally, Dr. Michael Forbes, a fellow in Pediatric Critical Care Medicine completed his training during this first year of funding and was the team leader on our study assessing the effect of HV in our model. He was the first author of the manuscript describing that work. Dr. Forbes is now Associate Director of the Pediatric Intensive Care Unit at Allegheny General Hospital in Pittsburgh.

(6) BODY

(a) Technical Objective 1: Mechanical ventilation strategies after severe TBI in rats

For over two decades, HV has been one of the most utilized strategies in the management of TBI. Laboratory and clinical studies, however, have verified that early after TBI, there is usually a state of reduced cerebral perfusion that may increase vulnerability to secondary injury. HV reduces intracranial hypertension by reducing cerebral blood volume; however, this generally is accompanied by a reduction in cerebral blood flow. A recent clinical study
suggested that HV may worsen outcome after TBI. However, in the field or during the initial stabilization, HV is often used (either planned or iatrogenically) and the first blood gas of patients in the emergency room can reveal significant hypocarbia. Using the CCI model in rats, we tested the effect of 4 h of aggressive HV (vs NV), beginning immediately after injury, on functional and neuropathological outcome.

Methods:
All of the protocols for the studies outlined below were approved by the Animal Care and Use Committee of the University of Pittsburgh. Intubated, mechanically ventilated, isoflurane-anesthetized male Sprague-Dawley rats (n = 26) were subjected to CCI (4 m/s, 2.5 mm depth of deformation) and randomized after 10 min to either HV [n = 13, PaCO2 = 20.3 ± 0.7 mm Hg] or NV [n = 13, PaCO2 = 34.9 ± 0.3 mm Hg] for 5 h. Beam balance and Morris water maze (MWM) performance latencies were measured in eight rats from each group on d 1-5 and 7-11 post CCI, respectively. Rats were killed at 14 d. Serial coronal sections of brain were studied for contusion volume and hippocampal neuron counting [CA1, CA3] by a blinded observer.

Results:
HV was readily achieved and could be sustained for 4 h in our model, and produced the anticipated systemic alkalosis (Figure 1). In addition, other variables could be tightly controlled for 4 h in our model (Figure 1).

![Graphs showing PaCO2, arterial pH, mean arterial blood pressure, and brain temperature over time](image)

*Figure 1. Entire time course of (A) PaCO2 (mm Hg), (B) arterial pH, (C) Mean arterial blood pressure (MABP, mm Hg), and (D) brain temperature (°C) in all rats treated with either NV (▲, n = 13) or HV (■, n = 13) after CCI. *p < 0.05 for NV vs HV. Data are mean ± SEM.*
Mortality rates were similar in both groups (2/13 vs 3/13, NV vs HV, respectively, NS). There were no differences between groups in mean arterial blood pressure, brain temperature, and serum glucose concentration. There were no differences between groups in either performance latencies for both beam balance (Figure 2) and MWM (Figure 3) or contusion volume (Figure 4).

**Figure 2.** Mean beam balance performance latencies (mean ± SEM, in sec) in rats before and on d 1-5 after CCI (4 m/s, 2.5 mm cortical deformation depth). Analysis of variance with repeated measures revealed no difference in duration of balance maintained between the two groups. (▲, NV, n = 8; ■, HV, n = 8). Data are mean ± SEM.
Figure 3. MWM performance latency to find a hidden platform (mean ± SEM, in sec) by rats on d 7-11 after CCI. There was no between group difference (▲, NV, n = 8; ■, HV, n = 8) when performances were compared using ANOVA with repeated measures. Data are mean ± SEM.
Figure 4. Graph depicting mean lesion area (left y-axis, mm\(^2\)) vs distance from occiput (mm) measured 14 d after CCI (NV, open bars, n = 11, HV, closed bars, n = 10). Contusion volume (mm\(^3\)) was calculated as the sum of these areas in each group and is depicted as cumulative volume (right y-axis) in the NV, △, and HV, ■ groups. There was no difference between groups in contusion volume (27.8 ± 5.1 vs 27.8 ± 3.1 mm\(^3\) NV vs HV, mean ± SEM).

However, in brain sections through the center of the contusion, hippocampal neuronal survival in HV reduced the number of surviving hippocampal CA3 neurons (29.7 [24.2 - 31.7] vs 19.9 [17.0 - 23.7] cells/high power field (NV vs HV, median [25\(^{th}\)-75\(^{th}\) percentiles] *p < 0.05, Mann-Whitney Rank Sum Test, Figure 5). In contrast to the detrimental effect on CA3 neurons, CA1 neuronal death was not increased by aggressive HV.
Figure 5. Box plots representing the number of surviving CA1 and CA3 hippocampal neurons in coronal brain sections through the center of the lesion in the hemisphere ipsilateral to the contusion. Cells were counted 14 days after injury. The median line is placed within the shaded 25th - 75th range. There was a reduction in the number of surviving CA3 hippocampal neurons after injury comparing NV and HV groups (29.7 [24.2 - 31.7] vs 19.9 [17.0 - 23.7], cells/high power field (hpf), *p < 0.05, Mann-Whitney rank sum test).

Conclusion:
Aggressive HV early after TBI augments CA3 hippocampal neuronal death; however, it did not impair functional outcome or expand the contusion. These data suggest that CA3 hippocampal neurons are selectively vulnerable to the effects of HV early after TBI. The injudicious application of HV early after TBI, such as in the field, may contribute to secondary neuronal injury.

Comment:
Hippocampal CA3 neurons are selectively vulnerable to delayed neuronal death after TBI. The mechanisms underlying this process remain speculative. Potential mechanisms include ischemia, TBI-induced excitotoxicity, apoptosis, and inflammation. We previously demonstrated that the hippocampus and cortex ipsilateral to the impact have marked flow reduction (at least 60%) at 2 h after TBI in the CCI model (2). CBF approaches ischemic levels in the core of the contusion at 2 h after injury. Although we have not evaluated the status of reactivity of the cerebral circulation to changes in PaCO₂ at 2 h after TBI in this model, we have reported that CO₂ reactivity is impaired, although still present (62-71% of baseline) in and around the contusion at 24 h after CCI in rats (3).
HV produces cerebral vasoconstriction and alkalosis. Alkalosis exacerbates N-methyl-D-aspartate (NMDA)-receptor mediated neurotoxicity. As a result of aggressive HV, the rats in our study were quite alkalotic as indicated by arterial pH measurements. Alkalosis appears to have deleterious effects on neurons. It could also be that the combined effect of alkalosis and further flow reduction by HV is deleterious in regions vulnerable to excitotoxicity such as CA3. Early, aggressive or prophylactic HV, therefore, in the context of reduced CBF, may exacerbate excitotoxic mechanisms and augment neuronal death.

Aggressive HV in the early low flow period did not worsen functional outcome or expand the contusion. The cognitive deficits in this model are modest. Indeed, to test therapies targeting an improvement in outcome, we may need a more severe injury (see below). Additional unilateral or bilateral hippocampal damage may be necessary to create more marked functional deficits. CA3 damage alone may not mediate post-TBI MWM deficits. However, hippocampal damage and memory deficits are common after TBI in humans, and exacerbation of neuronal death in any brain region would be highly undesirable.

This study does not completely address the uncommon situation where early after severe head injury marked intracranial hypertension is observed. HV may, in fact, be life saving in the setting of impending herniation. Similarly, we did not measure ICP or titrate ventilation to control cerebral perfusion pressure, and we evaluated only one level of HV and injury severity. We did not attempt to model the clinical scenario of optimal titration of ventilation when ICP is increased. Rather, we chose to evaluate the field setting and apply the worst case scenario, aggressive HV during the early post-trauma period when flow is already low and excitotoxicity is peaking. Our study does, however, show that HV is associated with a tangible risk to vulnerable neurons. To our knowledge, this is the first in vivo study demonstrating that HV can augment neuronal injury after TBI. This suggests that there is indeed a trade-off associated with this intervention.

**Recommendation**

We have shown that aggressive, early HV after TBI augments neuronal death in CA3 hippocampus in a rat model of cerebral contusion. The further reduction of CBF with HV during the low cerebral blood flow state immediately after severe TBI coupled with alkalosis may increase the vulnerability of selected neurons to damage. The results of this study reinforce the meticulous attention necessary to prevent secondary injury after TBI. A risk in the use of HV is demonstrated. Unless signs of impending herniation (unilateral pupillary dilation, hypertension, bradycardia) are present, this study supports the targeting of normocarbia for mechanical ventilation in the emergency stabilization of the brain trauma victim.

**(b) A field scenario of severe TBI for the evaluation of therapies proposed in Technical Objectives 2-4.**

Using our standard CCI model, neuronal death in selectively vulnerable regions and MWM deficits are present but modest. In rat model, we can nicely demonstrate exacerbation of damage with a deleterious strategy, namely HV. However, in technical objectives 2-4, our goal is to define strategies (hypothermia, anesthetics, anti-excitotoxic therapies) that will mitigate damage. Thus, the severity of damage must be increased, both from the standpoint of both hippocampal neuronal death and MWM deficit, to achieve this goal. Recently, in studies separate from this application, we published a variation of our CCI model that was designed to
increase the amount of hippocampal damage without totally destroying the hippocampus (and making it impossible to resuscitate)(4). This was achieved by adding a 30 min period of moderate hypoxemia (FiO₂ = 0.11) which also results in accompanying mild hypotension. This mimics the secondary insults in head injury victims so commonly seen in the field. In addition, in studies by our group separate from this application (5), we reported that both necrotic and apoptotic neuronal death is seen in this new variant of the CCI model. To be certain that this model would be suitable for technical objectives 2-4, it was essential to determine if the insult was accompanied by a significant MWM deficit.

Methods:
All of the protocols for the studies outlined below were approved by the Animal Care and Use Committee of the University of Pittsburgh. Intubated, mechanically ventilated, isoflurane-anesthetized male Sprague-Dawley rats (n = 20) were subjected to CCI (4 m/s, 2.5 mm depth of deformation) to the left parietal cortex using either a vertical or angled impact. Immediately after injury, the FiO₂ was reduced to 0.11 (inhalational anesthesia maintained constant by the addition of N₂ to the ventilator circuit). At 5 min after reducing the FiO₂ and at the completion of the 30 min secondary hypoxic insult, a blood gas is obtained to document the level of hypoxemia achieved, and then the FiO₂ is increased. Shams were subjected to all surgical procedures, but no insult (i.e., neither CCI nor hypoxemia). After the recovery periods, catheters were removed and anesthesia was discontinued. Rats were weaned from mechanical ventilation, extubated, and returned to their cages until further study. Motor and cognitive outcome were assessed as previously described.

Results:
Both vertical and angled impacts resulted in significant motor and cognitive deficits as assessed by beam balance, and MWM paradigms (Figures 6, and 7, respectively).

![Figure 6. Mean beam balance performance latencies (mean ± SEM, in sec) in rats before and on d 1-5 after either vertical or angled CCI with secondary hypoxic insult. Analysis of variance with repeated measures revealed no difference in duration of balance maintained between the two insults. Both insults were significantly different from sham.](image-url)
Figure 7. MWM performance latency to find a hidden platform (mean ± SEM, in sec) by rats on d 14-21 after either vertical or angled CCI with secondary hypoxemic insult. Analysis of variance with repeated measures revealed no difference between the two insults. Both insults were significantly different from sham.

Conclusion:
Combined with our prior publications showing both a well defined contusion and neuronal death by both apoptosis and necrosis in this model (4,5), the functional deficits produced with either a vertical or angled insult set the stage for studies proposed in Technical Objectives 2-4. We have chosen to use the vertical insult with hypoxemia, since all of our initial studies with HV used a vertical impact. These will be addressed in years 2-3 of the funding period. In accordance with this plan, we are currently evaluating the effect of hypothermia in this model of CCI with a secondary insult (as outlined in Technical Objective #2). We plan to address Technical Objective 2 and part of Technical Objective 3 in funding year 2.

(7) CONCLUSION

In our work during the first year of funding addressing Technical Objective #1, we demonstrated that aggressive HV early after TBI augments CA3 hippocampal neuronal death. These data suggest that CA3 hippocampal neurons are selectively vulnerable to the effects of HV early after TBI. The injudicious application of HV early after TBI, such as in the field, may contribute to secondary neuronal injury. As previously discussed, the results of this study reinforce the meticulous attention necessary to prevent secondary injury after TBI. A risk in the use of HV is demonstrated. Unless signs of impending herniation (unilateral pupillary dilation, hypertension, bradycardia) are present, this study supports the targeting of normocarbia for mechanical ventilation in the emergency stabilization of the brain trauma victim. Finally, by adding a secondary insult to our injury model, we have set the stage to address the optimal
application of treatments to improve outcome as outlined in Technical Objectives 2-4, and those studies are underway.

(8) REFERENCES


October 23 1997

Patrick M. Koshanek, M.D.
Safer Center for Resuscitation Research
3434 Fifth Avenue
Pittsburgh, PA 15260

RE: Manuscript #8160

Dear Doctor Koshanek:

Thank you for your responsive revision of the paper entitled Hyperventilation early after controlled cortical impact augments neuronal death in CA3 hippocampus. It is now accepted for publication in the Journal of Neurosurgery.

Please send us an original revised title page that includes full names of all co-authors (first, middle and last) at your earliest convenience. We will then proceed with publication. We look forward to receiving the completed copyright form.

Our editorial staff will be in contact with you as your manuscript is prepared for publication. We would appreciate your timely response to queries that we have, as this plays an important part in determining to which issue your paper is assigned.

This is an important and excellent study. Thank you for sending it to us.

Sincerely,

John A. Jane, M.D., Ph.D.
Editor
Journal of Neurosurgery

JAJ:mas
Hyperventilation Early After Controlled Cortical Impact 
Augments Neuronal Death in CA3 Hippocampus

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Running Head: Hyperventilation after TBI augments neuronal death

Key Words: Head injury, rat, hyperventilation, alkalosis, hippocampus

Manuscript: #8160
Abstract

Introduction: Minimizing secondary injury after severe traumatic brain injury (TBI) is the primary goal of cerebral resuscitation. For over two decades, hyperventilation (HV) has been one of the most utilized strategies in the management of TBI. Laboratory and clinical studies, however, have verified a post-TBI state of reduced cerebral perfusion that may increase vulnerability to secondary injury. In addition, a clinical study suggested that HV may worsen outcome after TBI. Using the controlled cortical impact (CCI) model in rats, we tested the hypothesis that aggressive HV applied immediately after TBI would worsen functional outcome, expand the contusion, and promote neuronal death in selectively vulnerable hippocampal neurons.

Methods: Intubated, mechanically ventilated, isoflurane-anesthetized male Sprague-Dawley rats (n = 26) were subjected to CCI (4 m/s, 2.5 mm depth of deformation) and randomized after 10 min to either HV [n = 13, P_aCO_2 = 20.3 ± 0.7 mm Hg] or normal ventilation [NV; n = 13, P_aCO_2 = 34.9 ± 0.3 mm Hg] for 5 h. Beam balance and Morris water maze (MWM) performance latencies were measured in eight rats from each group on d 1-5 and 7-11 post CCI, respectively. Rats were killed at 14 d. Serial coronal sections of brain were studied for contusion volume and hippocampal neuron counting [CA1, CA3] by a blinded observer.

Results: Mortality rates were similar in both groups (2/13 vs 3/13, NV vs HV, respectively, NS). There were no differences between groups in mean arterial blood pressure, brain temperature, and serum glucose concentration. There were no differences between groups in either performance latencies for both beam balance and MWM or contusion volume (27.8 ± 5.1 mm^3 vs 27.8 ± 3.3 mm^3, NV vs HV, respectively, NS). In brain sections through the center of the contusion, hippocampal neuronal survival in the CA1 region was similar in both groups; however, HV reduced the number of surviving hippocampal CA3 neurons (29.7 [24.2
- 31.7] vs 19.9 [17.0 - 23.7] cells/high power field, NV vs HV, median [25th-75th percentiles] *p < 0.05, Mann-Whitney Rank Sum Test).

**Conclusion:** Aggressive HV early after TBI augments CA3 hippocampal neuronal death; however, it did not impair functional outcome or expand the contusion. These data suggest that CA3 hippocampal neurons are selectively vulnerable to the effects of HV after TBI. Further studies delineating the mechanisms underlying these effects are needed. The injudicious application of HV early after TBI may contribute to secondary neuronal injury.
Introduction

Traumatic brain injury (TBI) is often complicated by malignant intracranial hypertension,\textsuperscript{31} which is associated with high mortality and has been managed using a combination of therapies including osmotherapy, diuretics, sedation, neuromuscular blockade, optimization of cerebral perfusion pressure, and hyperventilation (HV).\textsuperscript{5,8,31,38,50} HV has been an integral part of the clinical armamentarium in the management of severe TBI for over 20 years.\textsuperscript{11} HV rapidly reduces cerebral blood flow (CBF) and cerebral blood volume (CBV) in areas of brain with intact CO\textsubscript{2} autoregulation, providing one therapeutic option in the management of TBI complicated by malignant intracranial hypertension.\textsuperscript{1,33,41}

Recent studies, however, have defined a state of reduced CBF early after TBI in humans\textsuperscript{2,30} and animals\textsuperscript{4,19,24,45,56,57}, particularly in the first 8 h after TBI. Some have hypothesized that the brain is more vulnerable to secondary injury during this period and that additional reduction of CBF by HV may attenuate the delivery of important energy substrates.\textsuperscript{6,11,29,37,46,47} Yoshida and Marmarou\textsuperscript{58} reported that HV produced relative ischemia in cat brain after fluid percussion injury, showing an increase in brain lactate and inhibition of recovery of the ratio of phosphocreatine to inorganic phosphate. Muizelaar et al\textsuperscript{39} also demonstrated a loss of brain interstitial bicarbonate buffer after sustained prophylactic HV in rabbits. It has been reported that HV after TBI in animals and humans can reduce CBF to what traditionally have been considered ischemic levels.\textsuperscript{7,23,41} However, defining the ischemic threshold in injured tissue is problematic.\textsuperscript{21,32} Muizelaar, et al\textsuperscript{37} reported that prolonged HV after TBI in humans may worsen functional outcome raising questions regarding the appropriate indications and timing for the optimum application of HV after TBI. Recent guidelines for the management of severe head injury published in the \textit{Journal of Neurotrauma}\textsuperscript{5} state that “in the absence of intracranial hypertension, HV (PaCO\textsubscript{2} ≤ 35 mm Hg) therapy should be avoided
during the first 24 h after severe TBI...,” although, “ HV therapy may be necessary for brief periods where there is acute neurologic deterioration...” Consistent with these guidelines, in the setting of acute neurologic deterioration, aggressive HV is used by both emergency and critical care personnel. In addition, in the initial stabilization of the brain injured patient, aggressive HV (planned or iatrogenic) occasionally occurs (in both the pre-hospital and acute care settings). The specific impact of HV during this early low flow period remains to be determined. Despite the availability of well-characterized rodent models of TBI, which reproduce the early posttraumatic reduction in CBF, the effect of aggressive HV on histopathologic and functional outcome have not, to our knowledge, been investigated.

Using a rat model of percussive focal contusion, we hypothesized that aggressive HV, beginning immediately after TBI and continuing for 5 h, would worsen functional outcome, expand the contusion, and promote neuronal death in selectively vulnerable hippocampal neurons.
Materials and Methods

Subjects and Study Groups

All experimental protocols used in this report were approved by the Animal Care and Use Committee of the University of Pittsburgh. Twenty-six virus-free Sprague-Dawley rats (346 ± 5 g) were studied. Food and water were continuously available in their home cages. Rats were randomly assigned after TBI to receive normal ventilation (NV) (n = 13, P$_a$CO$_2$ = 30-40 mm Hg) or HV (n = 13, P$_a$CO$_2$ = 15-25 mm Hg).

Surgery and Brain Trauma Model

Anesthesia was induced using 4% isoflurane (Anaquest; Memphis, TN) in N$_2$O:O$_2$ (2:1). The rats were endotracheally intubated and mechanically ventilated. The isoflurane concentration was reduced to 2% followed by sterile surgical placement of a femoral arterial catheter for continuous mean arterial blood pressure (MABP) and arterial blood gas (ABG) monitoring. Penicillin 100,000 U IM (Upjohn, Kalamazoo, MI) and gentamicin 10 mg/kg IM (Elkins-Sinn; Cherry Hill, NJ) were given to minimize infection risk. Pancuronium bromide was administered 0.1 mg/kg/h via the arterial line (Elkins-Sinn; Cherry Hill, NJ) to control ventilation. Core temperature was monitored using a rectal probe.

After stereotactic head positioning (David Kopf; Tujunga, CA), the scalp was retracted, exposing the left parietal bone. A craniotomy was made using a high speed dental drill under a binocular operating microscope. A burr hole was made 5.0 mm anterior and 2.0 mm lateral to the bregma in the left skull and a temperature probe (0.009 in. outer diameter, Physitemp Corp., Clifton, NJ) was inserted through the burr hole 2 mm into the left parietal cortex. The bone flap was left in place and the isoflurane was reduced to 1.0% followed by a 30-min equilibration period. The brain
was maintained at $37 \pm 0.5^\circ C$. Normal ABGs were achieved in all rats and $P_aO_2$ was maintained $> 70$ mm Hg.

TBI was produced using a controlled cortical impact (CCI) device as recently described with minor modifications. Fifteen minutes before CCI, an arterial blood sample was obtained for measurement of ABG, glucose concentration, and hematocrit. The bone flap was removed and a vertical CCI (4 m/s impactor velocity, 2.5 mm deformation depth) was delivered onto the exposed dura overlying the left parietal cortex. The bone flap was replaced and sealed with dental cement and the scalp was resutured.

**Study Design**

The study protocol was designed to mimic the aggressive use of HV (vs NV) in the immediate post-trauma period in the prehospital as well as early hospital setting. Ten min after CCI, rats were randomized to either the NV group ($n = 13$, $P_aCO_2$ range, 30-40 mm Hg) or the HV group ($n = 13$, $P_aCO_2$ range, 15-25 mm Hg). The ventilator was adjusted to maintain normocarbia or hypocarbia for 5 h after CCI. ABGs were obtained at 30 min post-CCI, then hourly. MABP was recorded every 30 min after CCI. Brain and rectal temperatures were recorded every 15 min.

At 5 h after CCI, anesthesia was discontinued. Temperature probes and the femoral artery catheter were removed and the rat was allowed to wean from mechanical ventilation over 1 h and was extubated. Time to extubation was recorded. After extubation, supplemental $O_2$ was administered for 30 min. Once fully recovered, the rat was returned to its cage with full access to food and water.

**Functional Outcome and Behavior Assessment**

**Beam Balance**
Vestibulomotor function was tested using the beam balance test\textsuperscript{13} in eight rats from each group. One hour before surgery, the rat was placed lengthwise on a 1.5 cm wide beam suspended above ground. The time the rat remained on the beam was recorded (up to 60 s). The rat was then removed from the beam and the procedure was repeated. Rats were considered trained when they remained on the beam for 3 consecutive periods of 60 s. Beam balance tests were also performed daily on d 1-5 after injury. Three trials were recorded and averaged each day for each rat.

MWM

Cognitive function was tested in the same eight rats from each group using a standard variation of the Morris water maze (MWM) paradigm.\textsuperscript{14,34} A pool 180 cm in diameter and 60 cm in depth was painted black and filled with water to a depth of 28 cm. A clear Plexiglas® platform 10 cm in diameter and 26 cm high (2 cm below the water surface) was used as the hidden goal platform. The pool was located in a 2.5 x 2.5 m room with numerous extra-maze cues (e.g., posters, pipes, bookcase) that remained constant throughout the experiment. Testing started 7 d post-CCI in order to avoid motor deficits. Rats underwent four trials per day for 5 consecutive days to assess spatial memory performance. Rats started a trial once from each of the four possible start locations (north, south, east, west). The order of starting location was randomized. The goal platform was positioned 45 cm from the outside wall and was placed in either the northeast, southeast, southwest, or northwest quadrant of the maze. The location of the platform was held constant for each rat. Rats were manually placed in the pool facing the wall and were given a maximum of 120 sec to find the hidden platform. If the rats failed to find the platform within 120 sec, they were placed on the platform by the experimenter. All rats were allowed to remain on the platform for 30 sec before being placed in a heated incubator between trials. There was a 4 min intertrial interval. All data were recorded with a video tracking
Histology

At 14 d after CCI (after completion of all of the functional outcome testing), the rats were anesthetized with 5% isoflurane and killed by perfusion fixation using 10% buffered formalin. The brains were removed and post-fixed at 4°C for a minimum of 1 week and then cryoprotected in sucrose and cut in 10μ-coronal sections at 1 mm increments from the occipital to the frontal lobe with a cryotome and stained with Cresyl violet.

Contusion Volume

Using a computerized image analysis system (MCID, Imaging Research; St. Catherines, Ontario, Canada) the margin of the contusion was outlined and the sectional area of the contusion at each 1 mm increment was calculated by a blinded observer (MLF). Contusion volume in each rat was calculated as the sum of these sections.

Hippocampal Cell Counting

Neuronal loss in hippocampal regions CA1 and CA3 pyramidal layers was quantified. A coronal section through the dorsal hippocampus underlying the area of contusion, approximately 2.6 mm posterior to bregma was used for analysis for each rat. The regions were visualized at 100x then localized and counted at 400x by a blinded observer (RSBC). Only complete cells with a clearly defined cell body and nucleus were counted. Surviving pyramidal CA1 and CA3 hippocampal neurons were counted in six separate 400x fields for each region in both hemispheres. Sections were excluded if the boundary of the contusion extended into the pyramidal layers of the hippocampus or if fixation artifact precluded accurate counting. Data are
reported as the average number of surviving neurons per high power field for the CA1 and CA3 hippocampal regions in both the ipsilateral and contralateral hemispheres.

**Statistical Analysis**

Survival was compared between groups using the Fisher's exact test. Between group comparison of physiologic parameters and beam balance and MWM latencies were made using one- or two-way analysis of variance (ANOVA) for repeated measures where appropriate and post-hoc tests with appropriate correction for multiple comparisons. Contusion volume was normally distributed and was compared between groups using the Student's t-test. Hippocampal neuronal survival in CA1 and CA3 was not normally distributed and was compared between groups using the Mann-Whitney Rank Sum test. Significance was defined as $p < 0.05$. 

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Results

Physiologic Parameters

Baseline and 30-min post-randomization physiologic data are presented for all measured parameters in Table. After randomization, there was a marked increase in pH and decrease in $P_aCO_2$ in the HV group (vs baseline, $p < 0.05$). HV was also associated with a small (12 mm Hg) increase in $P_aO_2$ compared with baseline ($p < 0.05$). This difference was attributable to the increased minute ventilation and mean airway pressure in the HV group. At no time were any of the rats hypoxemic ($PaO_2 < 100$ mm Hg). The entire time course of arterial $PaCO_2$, arterial pH, MABP, and brain temperature after TBI is given for both groups in Figures 1a,d, respectively. Arterial $PaCO_2$ and pH differed between groups at all time points after randomization ($p < 0.05$). MABP and brain temperature were similar in both groups.

Five of 26 rats died during the 14-d study. All deaths occurred on the day of injury. Two rats remained unresponsive, were unable to demonstrate any spontaneous respiratory effort for 1 h after discontinuation of anesthesia, and were euthanized. Three rats developed pulmonary edema and/or respiratory distress and died early post-extubation. There were no between group differences in mortality (2/13 vs 3/13, NV vs HV, respectively). There were no between group differences in time to extubation (Table).

Functional Outcome Assessment

Beam balance

There was no difference between groups in motor performance latencies over time ($F_{1,15} = 0.17, p < 0.69$, Figure 2). Maximal impairment of performance occurred on d 1 or d 2 in both groups, and eventually returned to baseline. Beam balance performance did not differ significantly between NV and HV groups.

MWM
There was no difference between NV and HV groups in the latency to find the hidden platform in the MWM test ($F_{1,15} = 0.50$, $p < 0.50$, Figure 3). In addition, there was a non-statistically significant trend ($t_{13} = 1.77$, $p < 0.065$) for the HV rats to swim slower than the NV rats ($30.8 \pm 1.0$ vs $35.4 \pm 2.1$ cm/sec).

**Histopathology**

**Contusion volume**

At the injury level selected for this study, contusion was generally restricted to the parietal cortex beneath the impact site. Contusion volume in both groups is shown in Figure 4. There was no difference between groups ($27.8 \pm 5.1$ mm$^3$ vs $27.8 \pm 3.3$ mm$^3$, NV vs HV) in this outcome parameter.

**Hippocampal cell counting**

Figure 5 shows the number of surviving neurons/high power field in the CA1 and CA3 regions of the dorsal hippocampus ipsilateral to the contusion. There were no differences in the number of surviving CA1 hippocampal neurons between groups after CCI. There was, however, a further reduction in the number of surviving CA3 neurons in the HV group after CCI compared with the NV group (29.7 [24.2 - 31.7] vs 19.9 [17.0 - 23.7] neurons/high power field, median [25$^{th}$ - 75$^{th}$ percentiles], NV vs HV, $p < 0.05$). Neuronal cell counts in the CA1 and CA3 regions in the hemisphere contralateral to the contusion did not differ in either the NV or HV groups (55.3 [52.1 - 59.0] NV CA1 and 57.3 [51.3 - 59.0] HV CA1; 40.0 [36.6 - 41.2] NV CA3 and 38.0 [33.0 - 41.7] HV CA3).
Discussion

In a model of CCI-induced focal contusion in rats, aggressive HV for 5 h after TBI augments neuronal death in CA3 hippocampus ipsilateral to the contusion. However, HV did not worsen motor function or cognitive outcome, as assessed using standard beam balance and MWM paradigms, respectively, and did not increase contusion volume.

Hippocampal CA3 neurons are selectively vulnerable to delayed neuronal death after TBI.\textsuperscript{10,18,48,52,53} The mechanisms underlying this process remain speculative. Potential mechanisms include ischemia, TBI-induced excitotoxicity, apoptosis, and inflammation.\textsuperscript{10,18,48}

Yamakami and McIntosh\textsuperscript{56,57} reported reduced CBF as early as 15 and 30 min after TBI. Using a piglet model of TBI, Pfenninger, et al\textsuperscript{145} reported CBF reduction as early as 5-min post-TBI. Some flows were in the range consistent with ischemia. We have previously demonstrated that the hippocampus and cortex ipsilateral to the impact have marked flow reduction (at least 60%) at 2 h after TBI in the CCI model.\textsuperscript{24} CBF approaches ischemic levels in the core of the contusion at 2 h after injury. Although we have not evaluated the status of reactivity of the cerebral circulation to changes in PaCO$_2$ at 2 h after TBI in this model, we have reported that CO$_2$ reactivity is impaired, although still present (62-71% of baseline) in and around the contusion at 24 h after CCI in rats.\textsuperscript{15}

HV rapidly reduces CBV and intracranial pressure (ICP).\textsuperscript{11} In some studies, this intervention has been associated with CBF values consistent with ischemia or brain tissue hypoxia.\textsuperscript{7,11,41,47} After global cerebral ischemia in dogs, HV did not increase neuronal death,\textsuperscript{55} however, brains were assessed at 8 h after reperfusion, and neuronal death may be delayed. While ischemia may be considered a contributing mechanism in the observed augmented neuronal death, ischemia alone is an inadequate explanation for our findings in light of the preservation of CA1 neurons.
Although CA1 neurons are known to be selectively vulnerable to ischemic injury, they were not affected by HV in this paradigm. Furthermore, in our model, CA1 neurons are more proximal to the point of impact in the cortex, compared with CA3 neurons. The lack of CA1 neuronal death in light of ischemic and (presumed) anatomic vulnerability mitigates against ischemia and primary injury as putative mechanisms of neuronal death in hippocampus in this model. One limitation in this study is that neuronal counting using traditional histologic methods may underestimate cell loss because of a loss of hippocampal volume. We did not use stereologic methods in this study. However, CA1 neuronal counts did not differ between groups and were equivalent to those observed in shams studied in our laboratory in prior published and unpublished work. In addition, comparisons were only made between injured groups within this study.

HV produces cerebral vasoconstriction and alkalosis. Alkalosis exacerbates N-methyl-D-aspartate (NMDA)-receptor mediated neurotoxicity. As a result of aggressive HV, the rats in our study were quite alkalotic as indicated by arterial pH measurements. Although we did not measure brain pH, a decrease in PaCO₂ immediately reduces brain interstitial pH. Although alkalosis appears to have deleterious effects on neurons, acidosis has been shown to have both beneficial and detrimental effects. Giffard et al and others reported a neuroprotective effect of acidosis via an attenuation of the NMDA receptor activation in vitro. Rosner and Becker reported a deleterious effect of tissue acidosis after experimental TBI in cats. The spatial distribution of brain pH around the contusion and in the hippocampus has not been determined for either NV or HV conditions in our model.

Finally, the potential effects of HV on other mechanisms such as post-traumatic seizures or axonal injury may contribute to the enhanced vulnerability of CA3 neurons. The lateralization of the deleterious effects also raises the possibility that spreading wave depression may be a component of the neurotoxic milieu after TBI.
in this model of focal contusion. It could also be that the combined effect of alkalosis and further flow reduction by HV is deleterious in regions vulnerable to excitotoxicity such as CA3. Early, aggressive or prophylactic HV, therefore, in the context of reduced CBF, may potentiate excitotoxic mechanisms and augment neuronal death.

Aggressive HV in the early low flow period did not worsen functional outcome or expand the contusion, failing to support a significant portion of our initial hypothesis. Ultimate contusion size, in CCI or other models of focal contusion, is relatively refractory to manipulation by a variety of interventions; however, application of hypothermia particularly prior to injury does reduce contusion volume in CCI. Although we chose rather aggressive HV in an attempt to produce a maximal effect, we did not test the effect of HV on a milder contusion, which may have been more manipulable to secondary insults. The contusion penumbra has not been clearly defined in either of the standard rodent TBI models (CCI of fluid-percussion) for any level of injury. It is possible that selectively vulnerable CA3 hippocampal neurons are the only potential target for a deleterious effect of HV in our model. However, the effect of HV on the survival of neurons in the dentate gyrus or hilus (all vulnerable to TBI) was not assessed.

Hippocampal damage and memory deficits are common after TBI in humans. This study did not reveal any added effect of HV on functional deficits as measured by beam balance and MWM latencies. A number of factors may have contributed to this. Our sample size may have limited statistical power. However, this sample size was adequate to detect the exacerbation of functional deficits by the addition of 30 min of moderate hypoxemia (PaO_2 of 40 mm Hg) in our model. Secondly, the cognitive deficits in this model are modest compared with previous reports. Bilateral hippocampal damage may be necessary to create more marked functional deficits. In addition, CA3 damage may not mediate post-TBI MWM deficits.
Finally, the specific functional outcome paradigm may not have the necessary sensitivity to detect subtle functional deficits. For example, more demanding MWM paradigms have been employed by other investigators. However, in support of the testing strategy used, our hypothesis was that HV would worsen functional deficits.

This study does not completely address the uncommon situation where early after severe head injury marked intracranial hypertension is observed. HV may, in fact, be life saving in the setting of impeding herniation. Similarly, we did not measure ICP or titrate ventilation to control cerebral perfusion pressure, and we evaluated only one level of HV and injury severity. We did not attempt to model the clinical scenario of optimal titration of ventilation when ICP is increased. In the clinical setting, some investigators have demonstrated a wide variety of beneficial effects of HV under those conditions, such as homogenization of CBF, normalization of cerebral glucose uptake, and improvement in autoregulation. Rather, we chose the worst case scenario, aggressive HV during the early post-trauma period when flow is already low and excitotoxicity is peaking. Our study does, however, show that HV is associated with a tangible risk to vulnerable neurons in the CCI model. To our knowledge, this is the first in vivo study demonstrating that HV can augment neuronal injury after TBI. This suggests that there is indeed a trade-off associated with this intervention.

Conclusion

We have demonstrated that aggressive, early HV after TBI augments neuronal death in CA3 hippocampus. The further reduction of CBF with HV during the low cerebral blood flow state immediately after severe TBI coupled with alkalosis may increase the vulnerability of selected neurons to traumatic injury. Further studies are needed to delineate the relative contributions of these mechanisms to the
observed effects. The results of this study reinforce the meticulous attention necessary to prevent secondary injury after TBI. A risk in the use of HV is demonstrated.

Acknowledgment

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References


22. Jenkins LW, Moszynski K, Lyeth BG, et al: Increased vulnerability of the mildly traumatized rat brain to cerebral ischemia. The use of controlled secondary ischemia as a research tool to identify common or different mechanisms contributing to mechanical and ischemic brain injury Brain Research 477:211-224, 1989


54. Takadera T, Shumiada Y, Mohri T: Extracellular pH modulates N-Methyl-D-aspartate mediated neurotoxicity and calcium accumulation in rat cortical cultures Brain Res 572 (1-2) 126-131, 1990


Table: Physiologic Values

<table>
<thead>
<tr>
<th></th>
<th>Normal Ventilation + CCI</th>
<th></th>
<th>Hyperventilation + CCI</th>
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<td>Baseline</td>
<td>Post-Randomization</td>
<td>Baseline</td>
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<td>pH</td>
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<td>7.53 ± 0.01*</td>
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<td>P₄CO₂ (mm Hg)</td>
<td>36.7 ± 1.1</td>
<td>34.9 ± 0.3</td>
<td>37.2 ± 0.9</td>
<td>20.3 ± 0.7*</td>
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<td>P₄O₂ (mm Hg)</td>
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<td>167 ± 4</td>
<td>168 ± 4</td>
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<td>Base Deficit (mMol/L)</td>
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<td>Serum Glucose (mg%)</td>
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<td>Hct (%)</td>
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<td>Time to extubate (min)</td>
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<td>28 ± 6</td>
<td>N/A</td>
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<td>Brain Temperature (°C)</td>
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<td>Rectal Temperature (°C)</td>
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<td>Mean Arterial Pressure (mm Hg)</td>
<td>129 ± 4</td>
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</table>

a All values expressed as mean ± SEM
* p < 0.05, 30 min post-randomization vs baseline
Controlled cortical impact (CCI)
Figure Legends

Figure 1. Entire time course of (A) PaCO₂ (mm Hg), (B) arterial pH, (C) Mean arterial blood pressure (MABP, mm Hg), and (D) brain temperature (°C) in all rats treated with either normal ventilation (NV, ▲, n = 13) or hyperventilation (HV, ■, n = 13) after controlled cortical impact. * p < 0.05 for NV vs HV. Data are mean ± SEM.

Figure 2. Mean beam balance performance latencies (mean ± SEM, in sec) in rats before and on d 1-5 after controlled cortical impact (CCI: 4 m/s, 2.5 mm cortical deformation depth). Analysis of variance with repeated measures revealed no difference in duration of balance maintained between the two groups. (▲, normal ventilation, n = 8; ■, hyperventilation, n = 8). Data are mean ± SEM.

Figure 3. Morris water maze performance latency to find a hidden platform (mean ± SEM, in sec) by rats on d 7-11 after controlled cortical impact. There was no between group difference (▲, normal ventilation, n = 8; ■, hyperventilation, n = 8) when performances were compared using ANOVA with repeated measures. Data are mean ± SEM.

Figure 4. Graph depicting mean lesion area (left y-axis, mm²) vs distance from occiput (mm) measured 14 d after controlled cortical impact (normal ventilation NV, open bars, ■, n = 11/ hyperventilation, HV, closed bars, n = 10). Contusion volume (mm³) was calculated as the sum of these areas in each group and is depicted as cumulative volume (right y-axis) in the NV, ▲, and HV, ■, groups. There was no difference between groups in contusion volume (27.8 ± 5.1 vs 27.8 ± 3.1 mm³ NV vs HV, mean ± SEM).
Figure 5. Box plots representing the number of surviving CA1 and CA3 hippocampal neurons in coronal brain sections through the center of the lesion in the hemisphere ipsilateral to the contusion. Cells were counted 14 d after injury. The median line is placed within the shaded 25th - 75th range. There was a reduction in the number of surviving CA3 hippocampal neurons after injury comparing normal ventilation (NV) and hyperventilation (HV) groups (29.7 [24.2 - 31.7] vs 19.9 [17.0 - 23.7], cells/high power field (hpf), *p < 0.05, Mann-Whitney rank sum test).
Figure 1
Forbes et al
Figure 3
Forbes et al

Latency to find hidden platform (sec)

Days after surgery

0 10 20 30 40 50 60 70 80 90 100
Figure 4
Forbes et al
Figure 5
Forbes et al