Severe hemodilutional anemia increases cerebral tissue injury following acute neurotrauma

Gregory M. T. Hare,1,2 C. David Mazer,1,2 James S. Hutchison,3,4 Anya T. McLaren,1 Elaine Liu,1 Alipasha Rassouli,1 Jinglu Ai,1 Rachel E. Shaye,3 Julia A. Lockwood,3 Cynthia E. Hawkins,5 Nancy Sikich,1 Kevin To,1 and Andrew J. Baker1

1Department of Anesthesia, Cara Phelan Centre for Trauma Research, Keenan Research Centre in the Li Ka Shing Knowledge Institute, University of Toronto, St. Michael’s Hospital, Toronto; 2Department of Physiology, University of Toronto, Toronto; 3Departments of Critical Care Medicine and Paediatrics and the Neuroscience and Mental Health Program, The Hospital for Sick Children, Toronto; 4Interdepartmental Division of Critical Care, Faculty of Medicine, University of Toronto, Toronto; and 5Division of Neuropathology, Department of Laboratory Medicine, The Hospital for Sick Children, Toronto, Ontario, Canada

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Hare GM, Mazer CD, Hutchison JS, McLaren AT, Liu E, Rassouli A, Ai J, Shaye RE, Lockwood JA, Hawkins CE, Sikich N, To K, Baker AJ. Severe hemodilutional anemia increases cerebral tissue injury following acute neurotrauma. J Appl Physiol 103: 1021–1029, 2007. First published June 7, 2007; doi:10.1152/japplphysiol.01315.2006.—Anemia may worsen neurological outcomes following traumatic brain injury (TBI) by undefined mechanisms. We hypothesized that hemodilutional anemia accentuates hypoxic cerebral injury following TBI. Anesthetized rats underwent unilateral TBI or sham injury (n = 7). Target hemoglobin concentrations between 50 and 70 g/l were achieved by exchanging 40–50% of the blood volume (1:1) with pentastarch. The effect of TBI, anemia, and TBI-anemia was assessed by measuring brain tissue oxygen tension (PbrO2), regional cerebral blood flow (rCBF), jugular venous oxygen saturation (SjvO2), cerebral contusion area, and nuclear staining for programmed cell death. Baseline postinjury PbrO2 values in the TBI and TBI-anemia groups (9.3 ± 1.3 and 11.3 ± 4.1 Torr, respectively) were lower than the uninjured controls (18.2 ± 5.2 Torr, P < 0.05 for both). Hemodilution caused a further reduction in PbrO2 in the TBI-anemia group relative to the TBI group without anemia (7.8 ± 2.7 vs. 14.8 ± 3.9 Torr, P < 0.05). The rCBF remained stable after TBI and increased comparably after hemodilution in both anemia and TBI-anemia groups. The SjvO2 was elevated after TBI (87.4 ± 8.9%, P < 0.05) and increased further following hemodilution (95.0 ± 1.6%, P < 0.05). Cerebral contusion area and nuclear counts for programmed cell death were increased following TBI-anemia (4.1 ± 3.0 mm2 and 686 ± 192, respectively) relative to TBI alone (1.3 ± 0.3 mm2 and 404 ± 133, respectively, P < 0.05 for both). Hemodilutional anemia reduced cerebral PbrO2 and oxygen extraction and increased cell death following TBI. These results support our hypothesis that acute anemia accentuated hypoxic cerebral injury after neurotrauma.

TRAVMATIC BRAIN INJURY (TBI) is the leading cause of death and severe disability in young adults and children (13, 29). Hypoxia and hypotension both contribute to secondary cerebral injury following TBI (7). However, the effect of acute anemia has not been established. Direct measurements of cerebral tissue oxygen tension have demonstrated that prolonged episodes of cerebral tissue hypoxia are associated with increased mortality (48, 50). Furthermore, systemic hypoxia has been demonstrated to increase the degree of secondary cerebral injury following neurotrauma in experimental and clinical studies (2, 7, 8, 23). These outcomes demonstrate that the brain is at risk of secondary injury following primary cerebral trauma. Hypoxia may augment cerebral injury following neurotrauma by promoting apoptosis (36).

Although the theory underlying regional cerebral oxygen supply and demand has been advanced for decades, the impact of normovolemic, normotensive, hemodilutional anemia on acute neurotrauma remains undefined. Decreased viscosity may optimize blood rheology during acute hemodilution. However, the associated reduction in blood oxygen content may limit regional cerebral oxygen delivery (46, 49). This issue is important to neurotrauma patients for three reasons: first, resuscitation with blood-free solutions is common following cerebral trauma (5); second, transfusion practices in critical care medicine have evolved such that physicians are targeting progressively lower transfusion thresholds (18); and third, hypoxic-ischemic insult is associated with worsened mortality and impaired functional outcomes following TBI (3). These concerns are underscored by a recent clinical analysis that suggests that patients with neurotrauma may have better outcomes if the hemoglobin concentration was maintained at a higher target with more blood transfusions (34). We hypothesize that acute hemodilution will reduce cerebral tissue oxygen tension and increase cerebral injury following acute neurotrauma. Target hemoglobin concentrations between 50 and 70 g/l were selected to test this hypothesis.

MATERIALS AND METHODS

Effect of TBI and Anemia on Brain Tissue Oxygen Tension and Regional Cerebral Blood Flow

Animal model. All animal protocols were approved by the Animal Care and Use Committee at St. Michael’s Hospital in accordance with the requirements of the Canadian Council on Animal Care. Male Sprague-Dawley rats (350–400 g, 11–12 wk old, Charles River, St. Constant, Quebec, Canada) were assigned to one of three groups: 1) TBI only (TBI, n = 7); 2) hemodilutional anemia (anemia, n = 8),...
or 3) TBI and anemia (TBI-anemia, n = 10). Animals were initially anesthetized with ketamine-xylazine (100 and 7.5 mg/kg ip, Parke-Davis/Bayer, Toronto, Ontario, Canada), and anesthesia was maintained with 2–3% isoflurane in 50% oxygen (Abbott, St. Laurent, Quebec, Canada). Following trephecotomy, rats were ventilated with a pressure-controlled ventilator (Kent Scientific, Litchfield, CT) to achieve normocapnia as determined by blood gas analysis (Radiometer ALB 500, London Scientific, London, Ontario, Canada). The right jugular vein (polyethylene 90), tail vein, and the tail artery (polyethylene 50) were cannulated to measure mean arterial blood pressure (MAP) and central venous pressure (CVP) and to perform hemodilution. Co-oxygen (Radiometer, OSM 3) and blood gas analysis were performed on arterial and venous blood. Animals were then placed in a stereotaxic frame (ADI Instruments, Harvard Apparatus, St. Laurent, Quebec, Canada), and their scalps were incised sagittally.

**Fluid percussion injury.** A unilateral 5-mm-diameter burr hole was trephined over the left hemisphere at the level of bregma 0.0 to 5.2 mm lateral to the sagittal sinus, to expose the intact dura. The trephined over the left hemisphere at the level of bregma 0.0 to 5.0, 2 mm lateral to the sagittal sinus, to expose the intact dura. The 2-atm impact also correlates to clinical levels of injury associated with multiple trauma and systemic hemorrhage. Hemodilution in the TBI rats. In the two anemia groups, acute normovolemic hemodilutional anemia was induced over 10 min by simultaneous exchange of 30 ml/kg of arterial blood (50% estimated blood volume of 60 ml/kg), withdrawn from the tail artery, with an equivalent volume of pentastarch (Pentaspan, Bristol-Myers Squibb Canada, St. Laurent, Quebec, Canada) infused via the jugular vein. Volume exchange was performed over 10 min using a programmable “push-pull” pump (PHD 2000, Harvard Apparatus). Both MAP and CVP measurements were interrupted during the volume-exchange period. Following completion of volume exchange, all parameters were recorded for an additional 30 min before killing the animal by anesthetic overdose (ketamine 100 mg iv, Parke-Davis, Toronto, Ontario, Canada). In each group, arterial blood gas samples were taken at baseline and after hemodilution at 15-min intervals.

**Effects of TBI and Anemia on Arterial and Jugular Venous Blood Gases**

Three different groups of rats were utilized for these studies. Rats were anesthetized and ventilated as above and assigned to one of three groups: control (sham–sham, n = 6), anemia without TBI (anemia, n = 8), and TBI plus anemia (TBI-anemia, n = 6). Animals were prepared as described above with the exception that cannulation of the right jugular vein (polyethylene 90) was performed in a retrograde

Table 1. Effect of hemodilution on co-oximetry and arterial blood gas measurements following TBI and anemia

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Sample</th>
<th>Hemoglobin Concentration, g/l</th>
<th>%Saturation</th>
<th>O2 Content, mmol/l</th>
<th>pH</th>
<th>PaCO2, Torr</th>
<th>PaO2, Torr</th>
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<td>15</td>
<td>Baseline</td>
<td>140±15</td>
<td>99.3±2.4</td>
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<td>142±9</td>
<td>99.1±3.1</td>
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<td>7.40±0.04</td>
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<td>206.4±34.3</td>
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<td>8.5±0.7</td>
<td>7.38±0.07</td>
<td>36.3±7.9</td>
<td>171.3±48.0</td>
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<td>3.2±0.9*</td>
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<td><strong>Anemia (n = 8)</strong></td>
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<tr>
<td>15</td>
<td>Baseline</td>
<td>144±11</td>
<td>97.5±3.9</td>
<td>8.7±0.8</td>
<td>7.33±0.07</td>
<td>37.2±10.0</td>
<td>152.8±34.4</td>
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<td>3.4±0.8*</td>
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<td>177.6±59.9</td>
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<td>3.3±0.8*</td>
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<td>155.6±38.1</td>
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<td>15</td>
<td>Baseline</td>
<td>143±12</td>
<td>97.5±3.9</td>
<td>8.7±0.8</td>
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</tbody>
</table>

Values are means ± SD. TBI, traumatic brain injury; PaCO2, arterial PCO2; PaO2, arterial PO2. *P < 0.05 compared with corresponding baseline.
manner to allow for sampling of jugular venous blood. Venous blood samples were obtained before and after hemodilution by gravity-induced flow. The first 200 ml of each sample was discarded to minimize the effect of hemococoncentration secondary to venous occlusion between samples. Arterial and venous blood gas samples were taken simultaneously at similar time intervals as described above. The difference in arterial and venous oxygen content was calculated by subtracting corresponding venous and arterial values.

Effects of TBI and Anemia on Cerebral Contusion Area and TUNEL-Positive Cell Counts

Male Sprague-Dawley rats were anesthetized, intubated (14-gauge Angiocath), and ventilated before being assigned to one of three groups: control (sham-sham, n = 3), TBI alone (TBI, n = 7), and TBI plus anemia (TBI-anemia, n = 7). In this experiment, a less severe hemodilution protocol was utilized (40% estimated blood volume) by exchanging blood for pentastarch via the tail artery and vein to achieve a final target hemoglobin concentration near 70 g/l. This change allowed 100% survival to 5 days. In initial pilot experiments, hemodilution to a hemoglobin concentration of 50 g/l resulted in a survival rate near 70% with most deaths occurring within 12 h of surgery. Animals were then recovered and provided food and water ad libitum for 5 days. After 5 days, the rats were reanesthetized (2–3% isoflurane) and then perfused with 4% paraformaldehyde via a left ventricular catheter before whole brain extraction. After fixation, brain tissue was paraffin embedded. Whole mount coronal sections (5 μm) were prepared. Four coronal sections were obtained through the site of impact at the stereotaxic positions of bregma -2.3, -3.3 (anterior contusion), and bregma -5.3, -6.3 (posterior contusion). These sections were stained with cresyl violet and contusion areas were calculated for each section in a blinded manner, as previously described (36) [total magnification = 100X: eyepiece (10X) × objective (10X), Leica DMLS Microscope, Wetzlar, Germany]. Microscopic images were captured and the area of contusion measured in the cortex of each rat ipsilateral to the site of injury using Image J image analysis software (version 1.37, National Institutes of Health, Bethesda, MD). The microscopists (JAL, RES) were blinded to the experimental group of the animals. The total cerebral contusion area was calculated by adding the contusion areas from each of the four histological sections for each rat. Two adjacent coronal sections (bregma -2.3 and -6.3) were utilized to assess the number of cells undergoing programmed cell death per coronal section in each rat as indicated by nuclear staining with terminal deoxynucleotidyl trans-
ferase biotin-dUTP nick-end labeling (TUNEL). TUNEL staining was performed utilizing a fluorescence in situ cell death detection kit as previously described (40) (Roche Applied Science, catalog no. 1684795, Laval, Quebec, Canada). TUNEL-positive immunofluorescent cells were counted in both injured and contralateral noninjured hemispheres of each coronal section at total magnification of 100× [eye piece (10×) × objective (10×), Olympus Provis Microscope]. Each cerebral hemisphere was divided into three sections, including 1) the contusion site, 2) the subcontusion cerebral cortex, and 3) the basal structures. Digital images were captured, and total TUNEL-positive cells were counted by blinded observers (ATM, RES). Patterns of TUNEL staining were compared, and the total number of TUNEL-positive nuclei were counted.

Statistical Analysis

Data are presented as means ± SD. Normal distribution of data was confirmed before statistical analysis was performed utilizing the SPSS software (SPSS, version 11.5.0). Physiological measurements (HR, MAP, CVP, PbrO₂, and rCBF) were analyzed with a repeated-measures factorial ANOVA. The main effects of group, time, and hemisphere were assessed, and any interaction effects were defined. For each cerebral tissue oxygen tension data, the baseline measurements for each cerebral hemisphere were held as covariates. Post hoc comparisons with Tukey’s test were performed if statistical differences were determined by ANOVA. Comparison of MAP, PbrO₂, and rCBF between groups was performed at 10 min (baseline) and after hemodilution at 40, 60, and 70 min. The total cerebral contusion area and the total number of TUNEL-positive nuclei were assessed as the primary outcomes of tissue injury using a Student’s t-test. A Bonferroni adjustment was performed when appropriate. Statistical significance was assigned at the level of P < 0.05.

RESULTS

Effect of TBI and Anemia on PbrO₂ and rCBF

With the exception of the PbrO₂ measurements, there were no differences in any arterial blood gas, co-oximetry, or physiological parameter between the three groups at baseline (Table 1, Figs.1–4.) After hemodilution, there was a small increase in the MAP and CVP, relative to baseline, immediately following hemodilution, in both the anemia and TBI-anemia groups (Fig. 2, P < 0.05). However, there were no differences in MAP or CVP between any of the experimental groups at any time point (Fig. 2). Following hemodilution, the hemoglobin concentration in the anemia and TBI-anemia groups decreased comparably to minimum values of 56 ± 15 and 52 ± 13 g/l, respectively (Table 1, P < 0.05). The blood oxygen content was also reduced to a similar level in both groups.

PbrO₂ values were significantly reduced following injury in the TBI and TBI-anemia groups (9.3 ± 1.3 and 11.3 ± 4.1 Torr) relative to the anemia group (18.2 ± 5.2 Torr, Figs. 1, 3, and 4, P < 0.05 for both). Following TBI without hemodilution, the PbrO₂ recovered slowly to a maximum value of 14.8 ± 3.9 Torr at 70 min (Fig. 1, P < 0.05), while the MAP and rCBF remained stable. In the TBI-anemia group, hemodilution resulted in a further reduction in PbrO₂ to 7.8 ± 2.7 Torr by 70 min postinjury in the injured cerebral hemisphere (Fig. 3, P < 0.05), and this was significantly lower than the TBI group with no anemia (Fig. 4, P < 0.05). In the anemia group, the PbrO₂ values remained relatively stable in both cerebral hemispheres following hemodilution (Fig. 3).

rCBF remained stable following TBI alone (Fig. 1). Following hemodilution, the rCBF increased significantly, relative to baseline, in both cerebral hemispheres, in both anemia and TBI-anemia groups (Figs. 3 and 4, P < 0.05 relative to baseline). However, there were no differences in the CBF response between anemia and TBI-anemia groups for either cerebral hemisphere (Fig. 4).

Effects of TBI and Anemia on Arterial and Jugular Venous Blood Gases

In the control group (no TBI or hemodilution), the arterial and venous co-oximetry and arterial blood gas measurements
Fig. 4. Brain oxygen tension was reduced by TBI and further reduced by hemodilution. Three groups were compared: TBI, anemia alone, and TBI-anemia. A: mean arterial pressure remained near 80 mmHg with no differences between the 3 groups. B: brain tissue oxygen tension decreased following TBI at baseline. Following hemodilution, the brain oxygen tension decreased further in the TBI-anemia group relative to both TBI and anemia-only groups at 60 and 70 min. C: cerebral blood flow remained stable following TBI but was increased following hemodilution in both the TBI-anemia and anemia-alone groups. Regional cerebral blood flow values are relative to a baseline of 1.0. *$P < 0.05, \text{anemia vs. TBI and TBI-anemia groups; } \#\#P < 0.05, \text{anemia vs. TBI-anemia; } \#P < 0.05, \text{TBI vs. TBI-anemia group.}$

remained stable without any significant changes over time (data not shown). Hemodilution resulted in comparable reductions in arterial and venous hemoglobin concentrations and oxygen contents in both the anemia and TBI-anemia groups, relative to baseline values (Table 2, $P < 0.05$ for both). Arterial oxygen saturations remained between 97% and 100% in both groups. The baseline jugular venous oxygen saturation ($\text{SjVO}_2$) was increased in the TBI-anemia group ($87.4 \pm 8.9\%$, Table 2, $P < 0.05$) relative to the anemia group without TBI ($69.1 \pm 6.4\%$). Following hemodilution there was a small transient rise in the $\text{SjVO}_2$ in the anemia group to $77.9 \pm 2.6\%$ and a larger increase in the TBI-anemia group ($95.0 \pm 1.6\%$) (Table 2, $P < 0.05$, relative to baseline and between groups). The $\text{SjVO}_2$ remained increased for up to 45 min in the TBI-anemia group relative to the anemia group following hemodilution (Table 2, $P < 0.05$ between groups) but was not different from the anemia group at 60 min. The arterial-venous oxygen content difference was significantly reduced in the TBI-anemia group relative to the anemia group (Fig. 5, $P < 0.05$).

**Effects of TBI and Anemia on Cerebral Contusion Area and TUNEL-Positive Cell Counts**

Control (no TBI or hemodilution) rats did not exhibit any evidence of cerebral contusion (cresyl violet) and had less than 25 TUNEL-positive cells per cerebral hemisphere (data not shown). There was a significant reduction in the hemoglobin concentration in the TBI-anemia group relative to the TBI group ($68 \pm 5$ vs. $140 \pm 3$ g/l, respectively, $P < 0.05$). In the TBI and TBI-anemia groups, two areas of cerebral contusion were identified within the cerebral cortex. One area was located immediately superficial to the impact site ($a$), and the second area was located deeper within the cerebral cortex ($b$) (Fig. 6). The contusion area was larger in the posterior sections at bregma $-5.3$ and $-6.3$ relative to the anterior sections at bregma $-2.3$ and $-3.3$ in both groups (Figs. 6 and 7). After 5 days recovery, the total contusion area in the TBI-anemia group was significantly larger than that measured in the TBI group that underwent sham hemodilution (4.1 $\pm 3.0$ vs. 1.3 $\pm 0.3$ mm$^2$, Fig. 7, $P < 0.05$). There was also a corresponding increase in the number of TUNEL-positive cells in the TBI-anemia group relative to TBI alone [686 $\pm 192$ vs. 404 $\pm 133$ nuclei per coronal section, respectively (Fig. 7, $P < 0.05$)]. The majority of TUNEL staining occurred in the cerebral cortex at the site of contusion or immediately inferior in the subcontusion region. Less staining was observed in the basal structures (Fig. 7). Very little TUNEL staining was observed in the contralateral uninjured cerebral hemisphere.

**DISCUSSION**

We have demonstrated that acute anemia induced by normovolemic hemodilution led to cerebral hypoxia and accentuated cerebral injury in an experimental model of neurotrauma. Following unilateral traumatic brain injury, acute normovolemic hemodilution reduced regional cerebral tissue oxygen tension, while $\text{SjVO}_2$ and rCBF were elevated, suggesting that impairment of oxygen extraction had occurred. Acute normovolemic hemodilution also increased cerebral cortical tissue contusion area and cell death following TBI as measured in brain sections stained with cresyl violet and TUNEL. We also demonstrated that cerebral oxygen tension is maintained in the normal range and that no cerebral injury occurred following hemodilution without TBI. These data suggest that the brain is selectively vulnerable to anemia following neurotrauma.

The relevance of our experimental results is supported by a subanalysis of a clinical trial that suggests that patients suffering from acute neurotrauma may benefit from a higher transfusion threshold (18, 34). In addition, the “Lund therapy” management protocol, which contains higher transfusion thresholds, may lead to improved outcomes following acute neurotrauma (12). A recent clinical study in patients following cardiopulmonary bypass has demonstrated that relatively small changes in cerebral tissue oxygen tension may have an important impact on patient morbidity (38). Acute hemodilution is associated with increased neurological injury and higher mortality following cardiopulmonary bypass in animal models and in both adult and pediatric patients (14, 20, 25). The results of these studies, in combination with other studies showing that
Table 2. Effect of hemodilution and TBI on arterial and venous co-oximetry and blood gas analysis

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Sample</th>
<th>Hemoglobin Concentration, g/l</th>
<th>%Saturation</th>
<th>O₂ Content, mmol/l</th>
<th>pH</th>
<th>Pco₂, Torr</th>
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<td>169.8±32.0</td>
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<td>52.3±5.4</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 compared with corresponding baseline values. †P < 0.05 compared with corresponding anemia values.

Fig. 5. Jugal venous saturation was abnormally high following hemodilution in rats with TBI. A: jugular venous oxygen saturation was higher in the TBI-anemia rats at baseline following TBI, and further increased following hemodilution, before returning to control values by 60 min. B: these changes were reflected in lower differences between arterial and jugular venous oxygen content observed in TBI-anemia rats following hemodilution, suggesting that cerebral oxygen extraction was reduced following TBI. *P < 0.05 vs. baseline, †P < 0.05 between groups.
Experimental studies suggest that the cerebral metabolic rate for oxygen (CMRO$_2$) is immediately increased following TBI (6, 30). However, this initial state of hypermetabolism rapidly evolves into a more sustained state of hypometabolism (10, 30), possibly secondary to impaired mitochondrial function (9, 21). Early cerebral hypermetabolism cannot fully explain the reduction in cerebral tissue oxygen tension observed following TBI because such hypermetabolism would be expected to reduce the SjvO$_2$. The existing literature suggests that there is a decrease in oxygen consumption after TBI. Nonetheless, reduction in blood oxygen-carrying capacity with severe hemodilution can lead to inadequate regional cerebral tissue oxygen delivery and increased tissue damage. After 60 min, the SjvO$_2$ returned to baseline, possibly because of compensatory mechanisms that restored oxygen extraction (49).

There are some limitations to this study. Only one level of trauma was evaluated as part of the experimental design. The level of TBI was chosen to approximate the level of clinical injury that is often associated with systemic hemorrhage. Although target hemoglobin values near 50 and 70 g/l were used, future studies will be required to establish the hemoglobin threshold at which secondary injury occurs following TBI and anemia. We did not measure intracranial pressure, which is important in the assessment of cerebral perfusion. We did measure rCBF, however, which may be a better index of cerebral perfusion. We used laser-Doppler flowmetry to measure red blood cell flux since it correlates strongly with CBF in previous studies in animal models (28, 33). Tissue oxygen tension and laser-Doppler flow probes were located in different regions of the brain to minimize the adverse effect of local tissue damage at the site of the oxygen electrode on rCBF measurements. Furthermore, comparable changes in cortical and caudate tissue blood flow have been demonstrated following hemodilution in another experimental study (43). Pentastarch was chosen for hemodilution because volume exchange with synthetic starch colloids has been previously...
of increased CBF, suggesting that reduced cerebral oxygen extraction within the cerebral microcirculation likely contributed to the observed reduction in cerebral tissue oxygen tension. The increase in cerebral tissue contusion area and TUNEL-positive cell counts suggest that anemia leads to secondary cerebral injury following TBI. The evolving practice of restricting blood transfusion in critically ill patients might not be appropriate for patients with TBI. Optimal transfusion thresholds for patients with TBI may be different from other critically ill patients. Further research is required to determine if maintaining a higher hematocrit in patients suffering from TBI would improve clinical outcomes.

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