Neuroprotective effects of erythropoietin on acute metabolic and pathological changes in experimentally induced neurotrauma

Laboratory investigation

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Object. Head trauma is a dynamic process characterized by a cascade of metabolic and molecular events. Erythropoietin (EPO) has been shown to have neuroprotective effects in animal models of traumatic brain injury (TBI). Acute in vivo mechanisms and pathological changes associated with EPO following TBI are unknown. In this study the authors compare acute metabolic and pathological changes following TBI with and without systemically administered EPO.

Methods. Right frontal lobe microdialysis cannulae and right parietal lobe percussion hubs were inserted into 16 Sprague–Dawley rats. After a 4- to 5-day recovery, TBI was induced via a DragonFly fluid-percussion device at 2.5–2.8 atm. Rats were randomized into 2 groups, which received 5000 U/kg EPO or normal saline intraperitoneally 30 minutes after TBI. Microdialysis samples for glucose, lactate, pyruvate, and glutamate were obtained every 25 minutes for 10 hours. Rats were killed, their brains processed for light microscopy, and sections stained with H & E.

Results. Erythropoietin administered 30 minutes after TBI directly affects acute brain metabolism. Brains treated with EPO maintain higher levels of glucose 4–10 hours after TBI (p < 0.01), lower levels of lactate 6–10 hours after TBI (p < 0.01), and lower levels of pyruvate 7.5–10 hours after TBI (p < 0.01) compared with saline-treated controls. Erythropoietin maintains aerobic metabolism after TBI. Systemic EPO administration reduces acute TBI-induced lesion volume (p < 0.05).

Conclusions. Following TBI, neuron use initially increases, with subsequent depletion of extracellular glucose, resulting in increased levels of extracellular lactate and pyruvate. This energy requirement can result in cell death due to increased metabolic demands. These data suggest that the neuroprotective effect of EPO may be partially due to improved energy metabolism in the acute phase in this rat model of TBI. (DOI: 10.3171/JNS/2008/109/10/0708)

Key Words • erythropoietin • microdialysis • neuroprotection • rat • traumatic brain injury

T raumatic brain injury is a major cause of disability and death in the US, resulting in ~ 52,000 disabilities each year. Such injuries are a continuous challenge for health systems and a burden for the families and the community in terms of monetary cost, suffering, and disability. The death rate from TBI has been lowered from ~ 50% in the 1970s to 30% today. The improvement in survival can be attributed to more rapid transportation of patients to emergency departments, avoidance of low blood pressure, more effective methods of resuscitation, and meticulous intensive care. More importantly, this reduction in deaths has been associated with a rise in the proportion of survivors with relatively normal cerebral function.

However, despite a better understanding of the neurochemical cascades triggered by mechanical trauma as well as technical advances in neuromonitoring, the improved outcome of patients with severe head trauma has reached what seems to be an unmodifiable plateau. A further decrease in the rates of death and disability may be possible if the secondary injury produced by TBI can be reduced or eliminated.

Erythropoietin was first identified as a hematopoietic

Abbreviations used in this paper: BBB = blood–brain barrier; CNS = central nervous system; CSF = cerebrospinal fluid; EPO = erythropoietin; FPD = fluid-percussion device; NF-κB = nuclear factor-κB; RBC = red blood cell; TBI = traumatic brain injury.
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cytokine influencing the survival and differentiation of immature RBCs. After EPO was characterized, it was cloned in 1985 and began to be used in the clinical setting. In adults the kidneys produce the majority of circulating EPO. In more recent years, a recombinant form of EPO called rHuEPO has been used to treat and manage anemia associated with chronic renal failure. This modern treatment has substantially improved the quality of life for many patients receiving dialysis.

During erythropoiesis, EPO binds to the EPO receptor expressed on immature RBCs. This coupling of ligand and receptor acts to inhibit apoptosis of immature RBCs by supporting their proliferation and differentiation into mature cells. Subsequently, EPO and its receptor were localized in several brain and spinal cord regions of humans and animals. These findings led to multiple studies of the effect EPO has on the CNS. Studies using different experimental in vitro and in vivo models have demonstrated that EPO protects against neurological injury. In 2000, Brines et al. first reported the neuroprotective effect of EPO by examining acute histological changes after either EPO or placebo administration following induced TBI.

To improve the understanding of this neuroprotective effect, we used a rat in vivo microdialysis model to examine the mechanisms underlying neuroprotection and the ability of EPO to prevent secondary injury after neurotrauma.

We demonstrate in this study that administration of EPO to rats after TBI improved and maintained their neurological basal aerobic metabolic state. This study compares the neuroprotective effect of EPO with placebo administered after induced TBI by examining acute histological changes. Furthermore, we use microdialysis to discover the in vivo mechanisms underlying neuroprotection by measuring acute release of glutamate, lactate, glucose, and pyruvate.

Methods

Study Groups

Sixteen Sprague–Dawley rats were blindly randomized into 2 groups, each containing 8 animals. The EPO group received 5000 U/kg EPO in 6 ml normal saline intraperitoneally 30 minutes after TBI, whereas the saline group received saline vehicle only.

Anesthesia Protocol

A mixture of ketamine and xylazine (150:30 mg/ml) was administered intramuscularly at 0.6 mg/kg for surgeries to place the cannula guide and the FPD Luer connector. The same anesthesia combination was used during TBI, with supplemental dosing at 0.3 mg/kg as needed throughout the procedure to keep the rat under deep anesthesia.

Placement of Intracerebral Cannula Guide

A sagittal incision was made over the scalp. Using a fenestrating 5-mm drill, a craniotomy leaving the dura mater intact was created in the right parietal bone 1 mm off the midline sagittal suture and 4 mm posterior to the coronal suture. A 1-mm fenestrating drill was then used to create a small craniotomy 2 mm off the sagittal suture and directly over the coronal suture. An intracerebral cannula guide and the Luer-Lok connector for the FPD were implanted. The microdialysis guide cannula was placed 2.5 mm ventral to the dura. A Luer hub was inserted at the parietal craniotomy and Superglue was used to seal the hub in place. Cranial dental cement was applied, and then bone wax was used to cover the open Luer connector.

Traumatic Brain Injury Model

After a 4- to 5-day recovery period, general anesthesia was induced, the rats were placed prone, and the bone wax was removed. Next, the DragonFly HPD-1700 FPD was attached to the Luer-Lok connector via a male–male fitting, and the microdialysis probe was inserted through the cannula guide following removal of the stylet. Artificial CSF was infused at 1.2 µl/minute and microdialysis samples were extracted. After 50 minutes, a pressure impulse of 2.5–2.8 atm was applied via an FPD for ~ 20 msec directly on the dura mater to produce TBI. Intraperitoneal injections of EPO (5000 U/kg mixed in 6 ml normal saline) or vehicle (6 ml normal saline) were given 30 minutes post-TBI.

Microdialysis Procedure

A microdialysis probe (CMA 600, CMA/Microdialysis) was inserted through the guide into the deep frontal lobe white matter. Artificial CSF was perfused at 1.2 µl/minute. After a 50-minute stabilization period, microdialysis samples were continuously collected on ice at 25-minute intervals and then frozen immediately at –60°C.

Data Collection and Analysis

Values for glutamate, lactate, pyruvate, and glucose levels from 16 samples collected over the course of 10 hours were determined using a CMA 600 Analyzer. Level changes for all measured chemicals are expressed as percent variations from the mean baseline. Additionally, level changes were compared between groups. All data are expressed as the mean ± standard error. The Student t-test was used to analyze the time-course data within the groups and in the comparison of study and control groups. Differences with a probability value of < 0.05 were considered statistically significant.

Histological Findings

After 10 hours of microdialysis collection, each anesthetized rat was overdosed using halothane and perfused via cardiac puncture with normal saline followed by 10% buffered formalin. Following perfusion, each brain was removed and postfixed for 4–5 days in the same fixative. After postfixation, each specimen was sliced into gross serial sections, processed for paraffin embedding, sectioned at 5 µm, and stained with H & E. Slides were examined by 2 pathologists who had no knowledge of drug or placebo groups. The area of the lesion volume was measured based on slices from the right parietal lobe according to the method of Statler et al. To avoid sampling bias, the stereology method of Howard and Reed was used.
Results

Glucose Levels After TBI

Glucose levels after TBI were maintained in rats administered EPO 30 minutes post-TBI. Figure 1A shows the levels of glucose measured after TBI in rats receiving EPO or normal saline 30 minutes post-TBI. Two baseline glucose samples were taken in 25-minute intervals prior to induction of TBI. The results yielded a baseline level that was similar in both groups (mean 0.07 mmol/L). Statistically the levels did not show a significant difference. After 50 minutes, TBI was induced using the Dragon-Fly FPD, delivering a fluid wave at ~ 2.5–2.8 atm. Rats experienced apnea for ~ 5 seconds and exhibited classic brainstem posturing in bilateral upper extremities and tail. Thirty minutes after TBI, 6 ml EPO in normal saline (5000/U/kg) or 6 ml normal saline was administered intraperitoneally. Glucose microdialysis samples from the right frontal lobe were then obtained. Immediately after TBI, glucose levels doubled in both groups, to ~ 0.013 mmol/L. After the initial peak, the glucose levels began to fall equally in both groups until the 4th hour of measurement. At this time, the EPO and saline groups began to separate. Higher glucose levels at 4–10 hours after TBI were observed in rats receiving EPO compared with saline-treated controls (p < 0.01). In EPO-treated rats, glucose levels remained significantly closer to baseline levels compared with saline-treated controls. Immediately after TBI, glucose levels remained significantly closer to baseline levels compared with saline-treated controls.

Lactate Levels After TBI

Lactate levels remained at baseline after the action of EPO. Figure 1B shows the levels of lactate measured after TBI in rats receiving EPO or normal saline. The baseline level is similar in both groups, with a mean of 0.06 mmol/L. Statistically the levels did not show a significant difference. Immediately after TBI, lactate levels remained near the baseline until Sample 13, taken at the 6th hour of measurement, when the 2 groups began to separate. Lower lactate levels at 6–10 hours after TBI were observed in rats receiving EPO compared with saline controls (p < 0.01). In EPO-treated rats, lactate levels remained significantly lower and closer to baseline levels compared with saline controls.

Pyruvate Levels After TBI

Pyruvate levels were significantly unchanged until Sample 13 at Hour 6, when we begin to see acute changes in pyruvate, leading to significantly lower levels after stabilization at Hour 7.5. Figure 1C shows the levels of pyruvate measured in rats receiving EPO or normal saline 30 minutes post-TBI. A mean baseline level of 5 µmol/L is similar in both groups. Statistically the levels did not show a significant difference. Immediately after TBI, pyruvate levels remained near the mean of 5 µmol/L until the 6th hour of measurement. At that time, the EPO group began to exhibit sporadic spikes; these spikes were seen only in the EPO group and continued until 7.5 hours post-TBI, when the pyruvate level began to stabilize. In contrast, the saline-treated animals showed a consistent rise in pyruvate levels to ~ 9 µmol/L 10 hours post-TBI.
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Glutamate Levels After TBI

Microdialysis levels of glutamate yielded a baseline level not significantly different between groups, and this remained unchanged over the course of the experiment regardless of administration of EPO or normal saline. Data are not shown.

Histological Findings

Three selected samples shown in Fig. 2 display the histological features of tissue obtained near the right parietal lobe where TBI was induced. In a photomicrograph obtained at low power (× 40), Fig. 2A shows the saline-treated control rats with obvious areas of lesion and significant surrounding edema. The inset, obtained at higher power (× 200), illustrates ischemic neurons with pyknotic nuclei surrounded by glial cells. Furthermore, Fig. 2B also shows the lesion area and early calcification in saline-treated control rats. These results were compared with groups receiving EPO. Figure 2C shows mild lesion and edema, but to a much lesser degree than in saline-treated controls. The inset (× 400) again shows pyknotic nuclei and ischemic changes, but to a lesser degree. These histological results were quantified by measuring the area of lesion in mm² from the right parietal cortex in 8 saline-treated control rats and 8 rats treated with EPO post-TBI. Figure 3 shows that rats receiving EPO 30 minutes post-TBI have a significant decrease in the area of lesion volume (p < 0.05). Gross histological examination showed that ischemic neurons, edema, and area of lesion all had decreased in rats receiving EPO compared with those receiving saline by 10 hours after TBI.

Discussion

Head trauma is a dynamic process characterized by cascades of metabolic, cellular, and molecular events. These events include diffuse axonal injury, ischemia, excitotoxicity, and oxidative stress. Some of the neurological injury that occurs at the moment of traumatic impact is very likely to be irreversible. However, biochemical processes set in motion by traumatic injury that worsen the ultimate neurological outcome may be able to be mediated.

The present experiments show that EPO given 30 minutes post-TBI enables rats to maintain higher levels of glucose, lower levels of lactate, and lower levels of pyruvate compared with saline-treated controls. As measured based on tissue sections, there is also a decrease in lesion volume 10 hours after TBI with a single treatment of EPO. These data show acute in vivo effects of EPO ad-

![Infarct Area Ten Hours Post-TBI](image)

Fig. 3. Bar graph of measurements taken from slides of the right parietal lobe in 16 rats used to characterize the area of lesion after TBI. Although the primary injury exists, a secondary injury appears to have been prevented and a significant decrease of the lesion area is noted in the rats 10 hours after receiving EPO compared with saline-treated controls (p < 0.05).
ministration on brain metabolism in an animal model of TBI. Our results suggest that EPO’s acute neuroprotective effect may be a result of maintaining adequate glucose levels in the neuronal microenvironment.

Metabolic Changes in TBI

Following TBI, neurons experience increased utilization and depletion of extracellular glucose, resulting in increased brain levels of lactate and pyruvate. Because of increased metabolic demands, this energy crisis can result in cell death by shifting the metabolic mismatch from aerobic to anaerobic metabolism. Under baseline conditions, sufficient brain tissue O$_2$ suppresses lactate output.\(^2,2^9\) Lactate accumulation represents a slowing of the citric acid cycle metabolic pathway within the mitochondria in favor of anaerobic utilization of glucose. Pyruvate, a product of the glycolytic breakdown of glucose, undergoes anaerobic conversion to lactate by lactate dehydrogenase.\(^3^0\) During this process, the reduced form of nicotinamide adenine dinucleotide is reoxidized to form nicotinamide adenine dinucleotide, which is necessary for the continuation of glucose breakdown to pyruvate. Thus, an O$_2$ delivery/demand mismatch, as it occurs in the first hours after severe TBI, initiates the glycolytic accumulation of extracellular lactate.\(^4^9\) Increased levels of lactate in the dialysate and even in the CSF of patients with severe head injury have been shown to correlate with poor outcome or clinical deterioration.\(^1^4,1^7,3^6,5^0\)

As expected, a significant decrease in glucose (4 hours post-TBI) and a significant rise in lactate levels (6 hours post-TBI) were seen in our model after TBI. As glycolysis progressed, a late increase (7.5 hours post-TBI) in pyruvate was also seen. However, when rats were given a single dose of EPO 30 minutes after TBI, baseline glucose levels were maintained. With adequate glucose available to injured parietal lobe neurons, one does not see the metabolic mismatch, and thus lactate and pyruvate remain low. These maintained glucose levels suggest that EPO may act directly to potentiate adequate brain perfusion and O$_2$ delivery to the cerebral cortex. Therefore, EPO appears to preserve aerobic metabolism after TBI.

Role of EPO in TBI

The efficacy of EPO in TBI has been evaluated in only 1 study in rodents, which was published by Brines et al.\(^5\) in 2000. Here blunt trauma to the temporal and frontal cortices was produced using a pneumatic piston. Rats were given EPO at different time points: before, concurrently with, 3 hours after, or 6 hours after TBI, and they continued to receive EPO or saline for a total of 5 days. Rats were allowed to survive for 5 additional days and then were killed. Using the MCID image analysis system, a quantitative analysis of injury volume was performed, and this showed that animals given EPO had a significant reduction of injury volume when compared with the saline-treated group. In addition, regions surrounding the necrotic core in EPO-treated animals were characterized by markedly reduced acute inflammatory infiltrate. Brines et al. also demonstrated the ability over time of systemically administered multiple doses of EPO to protect brain tissue from blunt trauma. Our study demonstrates that this effect can be seen at 10 hours post-TBI by using a single dose of EPO. By its maintenance of aerobic metabolism and O$_2$ delivery, EPO given 30 minutes post-TBI may be indirectly responsible for the decreases seen in the gross amount of lesion volume and cytotoxic edema produced after TBI.

Mechanism of EPO in the CNS

The overall in vivo mechanism of EPO’s effect after different types of brain injury has begun to be explored. Current literature suggests that EPO also offers protection against necrosis and apoptosis in vivo models of induced stroke, neurotrauma, experimental autoimmune encephalitis, kinase-induced seizures, subarachnoid hemorrhage, and ischemic spinal cord trauma.\(^1^4,5,7^–,1^0,1^8^–,2^1,2^6^–,2^8,3^5,4^0,4^2^–,4^4,4^6\) These studies begin to show that EPO exhibits a neuroprotective effect in several types of cerebral macro- and microcirculatory dysfunction.\(^5,6\)

The manner in which systemically administered EPO acts or enters the CNS remains a matter of controversy. The action of EPO has been demonstrated by both intrathecal and systemic administration. Numerous studies have characterized EPO and EPO receptors present in animal and human brains.\(^3^7\) Further studies have shown that the EPO gene is expressed in human, monkey, and mouse brains.\(^1^2,3^2\) Brines et al.\(^5\) have recently suggested that EPO may be transported across the BBB by a specific receptor-mediated mechanism. They characterized this mechanism by systemic administration of EPO in uninjured animals and found translocation of peripheral EPO across the BBB. Thus, it is widely accepted that systemically administered EPO crosses the BBB.

Many specific mechanisms have been proposed through which EPO could achieve neuroprotective effects after TBI, but to date no acute in vivo metabolic mechanism has yet been described. First, EPO protects neurons from glutamate toxicity by activation of excitatory Ca$^{++}$ channels\(^4^0\) and appears to increase antioxidant activity in neurons.\(^4^3\) Erythropoietin has also been shown to decrease apoptotic activity in endothelial cells,\(^4\) and it appears to prevent neuronal apoptosis by the activation of Janus kinase–2 and NF-κB–signaling pathways.\(^4^6\) Furthermore, EPO binds to its receptor, which directly results in the activation of Janus kinase–2. This activation results in a downstream cascade, which begins with phosphorylation of the inhibitor of NF-κB. The phosphorylation event results in subsequent translocation of the transcription factor NF-κB from the cytoplasm to the nucleus, and eventually transcription of neuroprotective and antiapoptotic genes.\(^4^7\) The EPO given after TBI may systemically cross the BBB and act directly on these channels. Furthermore, EPO appears to effect angiogenesis in the ischemic brain.\(^4^8\) With increased angiogenesis, blood flow and tissue oxygenation in the border zone of the ischemic area are maintained.\(^4^5\) These mechanisms may be the underlying route in which EPO not only acutely increases blood flow and tissue oxygenation but begins to offer protection from early apoptotic activity and prooxidants. The coupling of these mechanisms with the ability to maintain glucose levels and prevent anaerobic metabolism will require further study. Promising evidence
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that early pharmacological intervention with EPO provides neuroprotection in the management of TBI may warrant a clinical trial in human patients. Thus, the knowledge obtained from our study may contribute to further improvement in TBI management and outcome.

One limitation of our study is that all rats were studied after induction of general anesthesia with ketamine and xylazine. Ketamine, like most anesthetics, has been suggested to have a neuroprotective effect.39 The extent to which ketamine might have protected the brain tissue from traumatic injury in our study is not known. However, reduced cerebral injury associated with the higher levels of glucose, and lower levels of lactate associated with EPO treatment are probably not due to the effect of ketamine, because both groups of rats received a similar amount of the anesthetic during the experiment.

Another limitation is that the long-term effect of EPO on glutamate, lactate, glucose, and pyruvate in TBI was not evaluated. However, it has been reported that administration of EPO enhanced neurogenesis and restored spatial memory in rats 14 days after traumatic injury,31,49 which suggests the long-term benefits of EPO in TBI. A further study is needed to examine the long-term effect of EPO on glutamate, lactate, glucose, and pyruvate in this rat model of TBI.

**Overall Findings**

Our study begins to provide a better understanding of the mechanisms involved in the neuroprotective effects offered by EPO in the setting of acute TBI. As the foregoing data illustrate, a single dose of systemically administered EPO maintains glucose levels and oxygen delivery to the cerebral cortex and helps stabilize aerobic metabolism. On histological examination, the volume of the lesion is significantly reduced and may be the result of decreased inflammation, increased antioxidant activity, increased oxygen delivery, and perhaps increased cerebral blood flow.

**Conclusions**

The mortality rate from severe TBI has decreased dramatically since the 1970s. If patients can be treated with agents that can prevent secondary brain injury, a further decrease in death and disability is possible. The present study demonstrates promising evidence that early pharmacological intervention with EPO provides significant neuroprotection against TBI in the rat model. This mechanism would imply, at least in part, an improved glucose metabolism in the affected brain.

**Disclosure**

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**References**


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