Beneficial effect of erythropoietin short peptide on acute traumatic brain injury

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EPO – erythropoietin

TBI – traumatic brain injury

CCI – controlled cortical impact

PBS – phosphate buffered saline

TUNEL - terminal deoxynucleotideyl transferase-mediated dUTP nick end labeling

ABSTRACT

There is currently no effective medical treatment for traumatic brain injury. Beyond the immediate physical damage caused by initial impact, additional damage evolves due to the inflammatory response that follows brain injury. Here we show that therapy with JM4, a low molecular weight 19 amino acid non-hematopoietic erythropoietin (EPO) peptidyl fragment, containing amino acids 28-46 derived from the first loop of EPO, markedly reduces acute brain injury. Mice underwent controlled cortical injury and received either whole molecule EPO, JM4, or sham-treatment with PBS. Animals treated with JM4 peptide exhibited a large decrease in number of dead neural cells and a marked reduction in lesion size at both 3 and 8 days post-injury. Therapy with JM4 also led to improved functional recovery and we observed a treatment window for JM4 peptide that remained open for at least 9 hours post-injury. The full length EPO molecule was divided into a series of 6 contiguous peptide segments; the JM4-containing segment and the adjoining downstream region contained the bulk of the death attenuating effects seen with intact EPO molecule following TBI. These findings indicate that the JM4 molecule substantially blocks cell death and brain injury following acute brain trauma and as such presents an excellent opportunity to explore the therapeutic potential of a small peptide EPO derivative in the medical treatment of TBI.

INTRODUCTION

Traumatic brain injury (TBI) affects up to 10 million people worldwide each year [1]. The health effects of traumatic brain injury can be debilitating and may result in long-term disability, yet there is currently no effective therapy for TBI. Thus, there remains a pressing need for the development of effective therapeutic agents to combat this condition.

The intact erythropoietin molecule (EPO) is a 165 amino glycoprotein cytokine that was initially identified as a hematopoietic growth factor and has been used extensively for anemia treatment in humans. Whole EPO received considerable attention over the past decade due to reports of its possible neuroprotective and neuroregenerative properties following CNS injury, including middle cerebral artery occlusion, focal ischemia, subarachnoid hemorrhage, and traumatic brain injury [2-6]. In several studies, EPO demonstrated efficacy in animal models of stroke and TBI [7-10]. For example, Wang et al. found that EPO aided in angiogenesis and the recovery of neurologic function following stroke injury in a rat animal model [9] and Brines et al. demonstrated that EPO treatment markedly reduced infarct volume in both ischemic and cortically concussed rat brain [5]. These studies have provided evidence to support EPO's promise as a beneficial treatment in an array of CNS disorders.

However, the side effect profile of whole-molecule EPO remains a clear disadvantage when administered for any extended period of time. A clinical trial in Germany using recombinant human EPO (rhEPO) as a treatment for stroke reported beneficial results *post hoc* in certain subgroups of patients despite unacceptably high mortality in patients treated with EPO [11]. Other studies have found that as EPO elevates hematocrit, side effects such as thrombosis and cardiovascular complications can arise [12, 13]. Harnessing the neuroprotective capabilities of rhEPO without unacceptable side effects brought about by its

hematopoietic effects could therefore lead to the development of an effective TBI treatment strategy.

In this study we designed a novel small, cyclical, EPO-derived peptide based on a non-hematopoietic domain within the early sequence (amino acids 28 – 46) of full-length erythropoietin molecule that we have named JM4. We evaluated its neuroprotective capabilities on a controlled cortical impact (CCI) model of traumatic brain injury in mice. Groups of injured mice were sham-treated with PBS, whole molecule EPO, or small EPO-derived peptide JM4 shortly after impact. Neural cell death in the injured brain lesion was quantified by TUNEL staining at 3 or 8 days following injury. In addition, we determined the therapeutic window for treatment following brain injury by delaying the initial post-injury dosage of JM4. The findings indicate that JM4 provided remarkable CNS protection from cell death and enhanced clinical recovery following acute traumatic brain injury.

MATERIALS AND METHODS

EPO and peptides

Whole EPO molecule (Epoetin Alfa, Ortho Biotech products, L.P.) in 2000 U/ml vial stock was stored at 4^{o}C. EPO-derived small peptide JM4 (N28-GCAEHCSLNENITVPDTKV-46C) was synthesized and purified by United Biochemical Research (Seattle, Washington). Peptides were synthesized using solid phase techniques and purified by high performance liquid chromatography (HPLC) to 90% purity. The identity and cyclic nature of JM4 were established by MALDI mass spectrometry. JM4 was dissolved in PBS (at 1 mg/mL) and kept in small aliquots at -20^{o}C until use.

Animals

Male C57BL/6 (8-10 weeks old) mice weighing 20-25g were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a conventional facility receiving standard diet and water *ad libitum*. The studies were conducted in accordance with the Animal Component of Research Protocol guidelines at the VA Medical Center, East Orange, NJ and with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Controlled cortical impact animal model

C57BL/6 mice were anesthetized with isoflurane/oxygen inhalation (1.5-2% isoflurane inhalation mixed in 21% oxygen) and placed in a stereotaxic frame with continuous anesthetic maintenance. Upon establishing the head position in the horizontal plane, a 4 mm craniotomy with the dura left intact over the cortex was produced lateral to the sagittal suture midway

between lambda and bregma. A cortical contusion injury was produced utilizing a pneumatically driven 2.5mm diameter rod tip at 3.5 m/s to a depth of 1 mm with a dwell time of 400ms (Precision System Instruments TBI-0300 Impactor, Lexington, KY). After injury, a 5mm disk constructed from dental cement was placed over the craniotomy site and adhered to the skull using cyanocrylate. During the early post-operative phase, animal temperature was maintained by placing the animals on heating pads. Sham-operated mice received a craniotomy but no cortical impact and the skull was also sealed with a dental cement plate. Mice were assigned randomly to treatment groups post-surgery.

Administration of EPO and JM4 peptide

Dose selection of JM4 was determined based on our previous dose response studies. Logarithmic doses below 10μg/animal were ineffective while doses above 10μg/animal failed to add any benefit. Intraperitoneal JM4 peptide (10μg/animal) was administered 15 minutes after TBI. For determination of the therapeutic window, treatment was delayed following TBI and the initial injection was given at 3, 9, or 24 hours. Mice in the intact whole-molecule EPO group received 5000 U/kg body weight rhEPO (Epoetin alpha, AMGEN, Thousand Oaks, CA) at 15 minutes post-TBI. JM4 peptide and EPO were diluted in 200μl of filtered sterile PBS prior to injection. Mice in the PBS and sham-operated groups received 200μl filtered sterile PBS 15 minutes post-TBI. All mice received subsequent additional JM4 treatments at 24 and 48 hours post-injury.

Blood brain barrier permeability of JM4

Anesthetized mice were injected intravenously (i.v.) through the tail vein with isotope labeled compound (^{3}H-JM4 or ^{14}C-inulin or both, 10μCi for each compound in total 100ul of PBS). 5 min after i.v. injection, we performed transcardiac perfusion with 150ml of heparinized saline to flush out the remaining free compound in the blood vessels. Whole brain was then removed, and rinsed briefly in cold saline. Tissue was weighed and transferred to 2mL of Tris-EDTA buffer, pH 7.4. Tissue was homogenized at max intensity for 1 min (kept cold on ice) and cooled for 10 min after. Protein was precipitated with 0.5mL trichloroacetic acid and centrifuged for 10 min at 2500g at 4°C. To pellets, 1mL of Tris-EDTA buffer was added and sonicated for 1 min with maximum intensity. The suspension was transferred to 5mL of scintillation cocktail before radioactivity was counted on a Beckman LS6500 machine (Indianapolis, IN). In our experiments, we injected mice with 10μCi of ^{3}H-JM4 and 10μCi of ^{14}C-inulin as a mixture or injected mice with 10μCi of ^{14}C-inulin alone. The radioactivity of ^{3}H and ^{14}C across different channels was concomitantly measured and we compared the ratio of ^{3}H/^{14}C in different tissues to determine movement across membranes.

Determination of the JM4 Therapeutic window

The initial dose of EPO-derived JM4 peptide was withheld for various time periods following brain injury for certain experiments. Following injury, groups of mice received their first dose of either 1) PBS (200μl) 15 minutes post-TBI; 2) JM4 (10 μg/mouse) 15 minutes post-TBI; 3) JM4 (10 μg/mouse) 3 hours post-TBI; 4) JM4 (10 μg/mouse) 9 hours post-TBI; or 5) JM4 (10 μg/mouse) 24 hours post-TBI. All mice received subsequent treatment doses at 24 hours and 48 hours following injury. Mice in the 24 hour delayed treatment group received a total of 2 doses rather than 3 doses of treatment. Mice were sacrificed 3 days post-TBI.

In situ TUNEL Detection and Cell Death Quantification

Mice were sacrificed at 3 days or 8 days post-injury and brains were collected and frozen immediately on dry ice for cryosectioning. Frozen brains were serially sectioned in coronal cuts onto 15 slides with 12 sections (16μm) per slide extending from the anterior to the posterior edge of the lesion. Sections were placed so that each individual slide contained a representative sampling of the entire lesion within the injured hemisphere.

Cell death in the mouse brains was detected using a modification of the Apoptag (Millipore, Billerica, MA) *in situ* terminal deoxynucleotideyl transferase-mediated dUTP nick end labeling (TUNEL) method. Serial 16μm cryosections were mounted and acetone-fixed for 10 minutes. The slides were rehydrated and post-fixed in 2:1 ethanol:acetic acid for 11 minutes at -20^{o}C. The slides were washed thoroughly after post-fixation, incubated with equilibration buffer for 10 minutes, then immediately reacted with working strength TdT enzyme for 60 minutes at 37^{o}C. The TUNEL reaction was detected using Cy3 anti-digoxin (1:200, Jackson ImmunoResearch) staining for 45 minutes.

Images were captured using a fluorescent Olympus (Center Valley, PA) BX60 microscope fitted with a Retiga (Surrey, BC, Canada) 2000R digital camera at 10x. The total number of TUNEL positive cells in lesioned hemisphere cryosections was quantified using digital imaging software IP lab 4.0 and labeled cells were quantified by two independent observers blinded to animal treatment groups. Four slides of injured brain (slides 1, 5, 10, 15) were quantified so that the 48 sections were representative of the entire lesion. Counting was accomplished by the computer software with adjustment of parameters on the Q-imaging quantification software to account for the intensity of the TUNEL stain.

The injury area containing TUNEL positive cells was determined at 10x magnification using the same images employed for the quantification of TUNEL positive cells. The area containing the positive cells was outlined by hand. All 12 cryostat sections on an individual slide were considered in determining the quantification of area. Quantification was performed using IP lab 4.0 software.

Assessment of 6 contiguous fragments of whole EPO peptide to isolate the TBI cell death blocking effect

Six peptide fragments comprising nearly the entire amino acid sequence of whole EPO were synthesized (United Biochemical Research, Seattle, WA). The peptide fragments, termed EP-P#1 to EP-P#6, started at the N-terminal side of EPO and each spanned 25 amino acids (AA), so that EP-P#5 ended at AA residue 125. EP-P#6 contained AA residues D136 – 166R. The experimental design was the same as the CCI protocol, including the cortical impact, administration of peptide (see Administration of JM4), TUNEL detection, and quantification.

Simple Neuroassessment of Asymmetric Impairment (SNAP) Score

Animals received CCI and were treated with PBS (n=6) or JM4 (n=6) or received sham surgeries (n=6) in which a craniotomy was performed with skull capping afterward but no CCI took place. TBI animals were assessed for clinical recovery on days 1-8 and on day 10 following TBI by two blinded independent evaluators. A modified version of the SNAP [14] test was used. This version excluded the visual field test and combined the gait/posture portion with the head tilt. The baton test was separated into two categories, speed and accuracy, with a maximum of 2 points each. SNAP scores of 0 indicated no deficit and the total possible score

(except baton) for each test was 5. The specifics of scoring are described in the table below (Supplement 1).

Statistical Analysis

The data (mean ± SEM) between groups were compared by one-way analysis of variance (ANOVA) tests, followed by Tukey's test for *post hoc* comparison of individual group means between PBS, rhEPO, and JM4 peptide treated animals. The analysis of composite SNAP clinical scoring utilized a mixed design study ANOVA with Tukey's post hoc test using a within subject factor of time and a between subject factor of treatment given (PBS, JM4, or whole EPO). A value of $P < 0.05$ was considered statistically significant. Figures include all individual data points, with each point representing the data collected from an experimental animal. Rectangular boxes around the data points were added for visual enhancement of the data ranges. Data analyses were performed using Prism 5.0 software.

RESULTS

EPO-derived JM4 peptide crosses the blood brain barrier to block cell death in traumatic brain injury

We determined that radiolabeled 3H-JM4 indeed passes the blood brain barrier. Simultaneous intravenous injections of 3H-JM4 and 14C-Inulin were performed in normal mice, followed by serum collection at 5 minutes post-injection and then a careful washout protocol and collection of the brain. Non-permeable 14C-Inulin served as a negative control. Data indicated brain/serum ratio of JM4 greatly exceeds that of inulin indicating JM4 likely crosses the BBB (Table 1).

To evaluate the effect of JM4 treatment on acute traumatic brain injury after cortical contusion, mice were either sham-treated with PBS, treated with JM4, or treated with whole EPO molecule 15 minutes following injury. Mice were treated for 3 days and sacrificed at 72 hours while others were sacrificed later at 8 days post-injury. The frozen brain tissue was serially sectioned and stained using the TUNEL assay to identify cells with nuclear DNA fragmentation, indicative of cell death. The stained tissues were visualized using fluorescent microscopy and TUNEL positive cells were quantified.

TUNEL staining revealed a drastic reduction in TUNEL positive cells in the injured hemisphere of animals treated with JM4 (**Fig. 1B**) compared to PBS-treated control animals (**Fig. 1A**). Animals treated with whole-molecule EPO served as a positive control and also exhibited sharply reduced numbers of TUNEL positive cells. Additionally, TUNEL-stained JM4-treated brains tended toward a restricted pattern of cell death confined to the immediate area of the lesion core while PBS control-treated tissue displayed prominent, widespread expansion of cell death outward from the core, often extending into the basal ganglia and hippocampus (**Fig.1A**).

Cell death quantification following brain injury

To quantify the amount of cell death within the injury area of each animal, brains were serially cryosectioned from anterior to posterior. The slides were visualized by fluorescent Cy3 detection and quantified. The injury zone in the lesioned hemisphere was quantified by following the TUNEL negative margin immediately outside of the TUNEL reactive zone. The contralateral hemisphere in each brain displayed rare or no TUNEL positive cells.

Both whole EPO and JM4 were found to significantly reduce quantities of TUNEL positive cells within the injured hemisphere of animals receiving therapy for 3 days (**Fig. 2A**, P <0.001). Whole EPO therapy produced a 60% reduction in cell death in comparison to animals receiving sham (PBS) treatment (P <0.001). JM4 therapy performed comparably, with a 65% reduction in number of TUNEL positive cells (P <0.001).

This neuroprotective effect was also seen in animals treated for 3 days with sacrifice delayed until 8 days post-injury (**Fig. 2B**, P <0.001, ANOVA). JM4 treatment elicited a 55% reduction in TUNEL positive cells (P <0.05). In contrast, TBI animals treated with whole EPO for 3 days and sacrificed at 8 days post-injury displayed a more limited, non-significant 30% reduction (P =0.091). Though cell death at 8 days post-injury was not significantly diminished by whole EPO treatment, a trend towards a positive treatment effect was still present.

To further assess the extent of lesion size within the injured hemisphere, the total area containing TUNEL positive cells in each injured brain was calculated. To accomplish this, one slide from each animal containing representative sections of the whole brain was TUNEL-stained and the TUNEL positive area was defined by manually tracing around the TUNEL positive margins of the lesion using IP Lab 4.0.

Treatment with either whole EPO or JM4 resulted in a major reduction in lesion size compared to PBS sham-treated animals (**Fig. 3A**, *P* <0.001, ANOVA). In JM4-treated animals sacrificed 3 days post-injury, TUNEL staining revealed a reduction of over 55% in lesion area compared to sham-treated animal lesions (*P* <0.001). A 45% reduction in lesion size was observed in whole molecule EPO-treated animals (*P* <0.01). In animals treated for 3 days but sacrificed at 8 days, JM4 continued to induce a significant reduction in lesion size compared to sham PBS-treated controls (**Fig. 3B**, *P* <0.05). However, animals treated with whole molecule EPO and sacrificed 8 days post-TBI failed to demonstrate a significant reduction in lesion area (*P* =0.07). As with total dead cell count, whole EPO treatment induced a positive, though not significant, effect on lesion area reduction.

Clinical assessment of impairment

We tried several strategies designed to consistently quantify neurological deficit without success in the early stages of our study. However, Shelton and coworkers introduced a new Simple Neuroassessment of Asymmetric Impairment (SNAP) test which we found quite useful in reproducibly quantifying neurological impairment in brain injured mice when tested by blinded observers [14]. To assess each animal's degree of clinical impairment, we developed a modified version of the SNAP test (Supplement 1). Animals were followed and assessed by blinded observers on days 1 through 8 and on day 10 post-TBI (**Fig. 4**). The data was assessed using a mixed design ANOVA and indicated a main effect on Days Post TBI $F(8,120) = 35.94$, $P < 0.001$ and a main effect on Treatment $F(2,15) = 507.94$, $P < 0.001$, as well as Days Post TBI x Treatment interaction $F(16,120) = 15.09$, $P < 0.001$. Tukey's posthoc test indicated that JM4 treated mice performed better than PBS treated mice in composite clinical scoring ($P < 0.05$) and exhibited significantly less clinical deficit. Mice receiving sham surgeries in which

only the craniotomy was performed but no cortical injury took place performed better clinically than either JM4 or PBS treated mice (P <0.05). The maximum attainable score was 29 which would indicate complete impairment in every category, but the highest individual score on any day among all animals throughout the experiment was 13. Typically mice ranged from 3 – 10 in scoring. Therefore, absolute reductions in SNAP clinical score ranged from 0 to 4 points on any given day.

Therapeutic window

To simulate situations where drug delivery to the patient following traumatic brain injury would not be immediately feasible, we determined the duration of the therapeutic window during which short peptide JM4 treatment was effective.

We withheld the initial treatment of JM4 peptide for 3, 9, or 24 hours post-injury. Mice with initial delay of treatment for 3 or 9 hours subsequently received additional JM4 at 24 and 48 hours post-injury. Mice that received their first dose 24 hours post-injury and a subsequent dose at 48 hours post injury did not receive a third. Mice were sacrificed at 72 hours post-injury and the effect of treatment delay was determined on the injured brain using TUNEL staining.

TUNEL staining revealed greatly depressed levels of cell death in mice treated 15 minutes post-injury with the standard 3-day JM4 regimen (50% decrease) compared to sham-treated brain injured controls (**Fig. 5**, P <0.01). Furthermore, mice receiving delayed initial treatment with JM4 peptide at 3 hours post-injury showed marked cell death attenuation (50% decrease) equivalent to mice treated 15 minutes post injury (P <0.01). Mice that were administered initial JM4 after a 9-hour delay post-injury still demonstrated slightly greater diminished number of TUNEL-positive cells (65% decrease) compared to sham-treated TBI

controls (P <0.001). Mice receiving no treatment until 24 hours post-injury displayed reduced cell death compared to controls (30%) but the treatment effect failed to achieve significance. The sample size for the 24-hour group was limited to 3 animals.

Neuroprotection is limited to early segments of the whole EPO molecule

Six contiguous peptide fragments spanning the bulk of the entire EPO sequence were synthesized. Each fragment consisted of about 1/6 of the primary structure of whole molecule EPO, and each fragment was tested for neuroprotection efficacy in our TBI animal model and subsequent cell death attenuation quantified by TUNEL stain. Following injury, mice were treated with either PBS, JM4 peptide, or one of the six EPO peptide fragments. TBI animals were treated with an initial dose 15 minutes post-injury and treated every 24 hours for a total of 3 doses. Some groups of JM4-treated mice expressed significantly diminished quantities of TUNEL-positive cells (**Fig. 6**, P <0.005) and another short EPO peptide pHBSP [15], an 11mer peptide comprised of select AAs from the hydrophilic face of the B helix of EPO (AA 56 – 82), also showed a substantial reduction in TUNEL positive cell numbers (P <0.01). The EPO fragment peptides were labeled EP-P#1 through EP-P#6, with each increasing number corresponding to a peptide derived from a region closer to the C-terminal of EPO. The complete sequence of JM4 lies within EP-P#2, and the sequences of HBSP and pHBSP (small peptide derivatives of EPO) lie partially within EP-P#3 and EP-P#4 [15, 16]. Of these EPO segments, EP-P#2 which contained the JM4 sequence demonstrated the most significant reduction in quantity of TUNEL-positive cells within injured brain relative to the controls. The EP-P#3 segment also induced a reduction in TUNEL-positive cells. Therapy with EP-P#5

demonstrated an appreciable though more marginal level of reduction in brain following TBI ($P < 0.05$).

DISCUSSION

The purpose of this study was to investigate the tissue-protective effects of a non-hematopoietic domain derived from whole molecule EPO on traumatic brain injury in a mouse animal model. Attenuated cell death in JM4-treated animals appears to correlate with reduced clinical deficit following traumatic brain injury. We also showed that despite initial treatment delay, JM4 boasted a potent therapeutic effect that extended for at least the first 9 hours following injury. Finally, the neuroprotective characteristics of whole EPO appear to be contained within the early portion of the intact EPO sequence. The most profound cell death blocking effects were restricted to the JM4 containing sequence, EP-P#2, and to a slightly lesser degree EP-P#3.

JM4 peptide represents a newer class of EPO derivative composed of a small peptide region derived from the AB loop of the whole EPO molecule. We aimed to define a region of the EPO molecule that retained therapeutic efficacy in neuroprotection yet eliminated the undesired hematopoietic interactions [17] that have severely restricted the use of whole EPO treatment in TBI. The JM4 sequence (N28-GCAEHCSLNENITVPDTKV-46C) was chosen for the presence of a disulfide bond capable motif comprised of two cysteine residues in the first loop of EPO, allowing for a cyclic structure in solution to enhance stability. Other EPO derivatives based on the same desire to eliminate undesired hematopoiesis have been studied for their use in brain injury, including helix B surface peptide and a pyroglutamate helix B peptide (known as HBSP or ARA290) [16]. HBSP and its analog pHBSP have been found to be tissue-protective in models of stroke [18], cardiovascular disease [19, 20], experimental autoimmune encephalomyelitis (EAE) [21], and TBI [15]. In our investigation, JM4 also significantly limited the size of the lesion in injured brains following traumatic brain injury (Fig. 3). We elected to measure cell death with TUNEL staining which has been used extensively to assess for cell

death following TBI [22], [23]. We additionally observed significant attenuation of cell death in brain lesions whether treated with JM4 or EPO, indicating equivalent levels of neuroprotection can be afforded by both whole molecule EPO and JM4 (Fig. 2). When the whole EPO molecule was divided into consecutive fragments, our data supports the notion that only early segments of the molecule contain the significant neuroprotective effects afforded by EPO (Fig. 6A). This neuroprotective region (AA 28-82) contains the sequences for JM4, HBSP, and pHBSP (Fig. 6B), and of note the second fragment (EP-P#2) which contains the JM4 sequence demonstrated the most profound cell death blocking effect. Our data contributes to a small yet steadily growing body of literature demonstrating neuroprotective properties of EPO and the potential for its small peptide derivatives to take on a prominent role in the treatment of brain injury.

Several human trials have revealed that whole EPO treatment may lead to significantly increased incidence of thrombotic events. More alarmingly, in a Phase II/III study on the use of rhEPO for treatment of stroke, the death rate among patients treated with rhEPO was elevated to 16.4%, compared to 9.0% in the placebo group (p = 0.01) [11]. These findings impacted a recently published clinical study investigating the use of EPO following TBI to such a degree that the initial 3 dose regimen of EPO was decreased to only a single initial dose of EPO after injury midway through the study [24]. The study failed to report significant benefit in neurological recovery in patients, but it is important to note that the dosage used was about 1/10 of the most effective clinical dose in animal TBI models and that more than half of enrolled patients had a Glasgow Coma Score 8 or lower (severely impaired). In contrast, in our animal model most mice were conscious, alert, and moving within 30 – 60 minutes of their TBI procedures and received a complete EPO 3 dose equivalent of JM4. From the data on mortality

we might gather that the concentration of EPO required in circulating plasma to elicit its neuroprotective effects may risk the activation of erythropoiesis and downstream side effects.

In response to the side effects brought on by EPO treatment, derivatives of the whole EPO protein including our compound, JM4, have been designed and studied in animal models [25-27]. The goal of design was to maintain the tissue protective capabilities of the EPO molecule while eliminating the erythropoietic characteristics that lead to the previously described complications. JM4 has been previously demonstrated to have no effect on hematocrit in animals [17].

We additionally developed a clinical correlate to follow the level of TBI deficit during the disease course following injury. Brain injured mice treated with JM4 showed fewer deficits than their PBS sham-treated counterparts during the post-TBI course (Fig. 4). Scores for injured mice were significantly higher compared to baseline scores set by sham-operated uninjured mice, allowing us to discount the effects of the craniotomy and other surgical factors unrelated to CCI. From our experience, SNAP provides a useful clinical correlate to track neurologic impairment and recovery [14]. Our results indicate that JM4 remains effective in limiting neurological deficit following injury over a 10 day trial. Interestingly, on day 2, the composite SNAP scores of both JM4 and PBS treated groups fell dramatically, only to increase again on day 3. TBI has been associated with delayed activation of CCL2 and CCL20 as well as secondary delayed activation of IL-1b and IL-4 in a rat CCI model, suggesting a temporal relationship between the cytokine profile, their targeted cellular infiltrates, and observed clinical deficits [28]. Exploration of EPO as an immunomodulatory agent has revealed that EPO reduces levels of pro-inflammatory cytokines TNF-α, IL-1β, INF-γ, IL-6, and ICAM-1 following EPO treatment in TBI and EAE [29-31]. Other studies in EAE and cerebral malaria have demonstrated that whole molecule EPO treatment can modulate a highly unstable autoimmune

environment into an immune system of relatively normal activity, limiting peripheral and CNS infiltration by CD4+ and CD8+ T cells, reducing number of mononuclear cells and dendritic cells while suppressing Th17 cells, IFN- ɣ and TNF mRNA [29-32]. In some studies EPO also induced elevation of IL-10, and FoxP3+ Treg cells, factors that contribute toward immune silencing.

An important and highly practical aspect of the design of novel therapeutics is the time frame in which the molecules maintain a high level of efficacy. Whole molecule EPO reportedly loses protective effectiveness 6 hours post-injury [5]. An increase in therapeutic window duration by a few hours could represent a significant advantage over existing therapies. Surprisingly, delaying initial treatment of brain injured mice with JM4 peptide for 9 hours seemed to reduce TUNEL cell death as much as immediate treatment with JM4 (Fig. 5). These findings demonstrate that the therapeutic window for EPO-derived JM4 treatment is open for at least 9 hours post-injury and likely extends further toward the 24-hour post-injury mark. Studies have observed that TNF-α levels peak between 3 – 8 hours following TBI [33, 34]. Though the role of TNF-α in TBI has not been fully elucidated, this response potentially indicates improved JM4 efficacy associated with more optimal inflammatory attenuation by slightly delayed initial therapy.

Though much optimism has surrounded the use of whole EPO and its derivatives in TBI, still little is known about possible mechanisms of action in neuroprotection. In a mouse model of EAE we found that EPO exhibits a strong immunomodulatory effect by inhibiting the proliferation of dendritic cell and antigen-specific T cell populations while downregulating MHC-II and proinflammatory cytokine (INF- ɣ, IL-2, IL-6, TNF-a) expression. Whole molecule EPO promoted the expansion of T regulatory cells while also causing a striking decrease in peripheral Th17 cell levels [29]. All of these effects occurred in the lymph nodes and spleen

(peripheral lymphoid system) as well as within the damaged target organ (spinal cord). This characteristic has also been retained in the JM4 molecule as we have demonstrated that JM4 suppresses IL-2, IL-5, IL-6, TNF-α, and IFN- γ while limiting peripheral mononuclear and dendritic cell populations in EAE-diseased animals [17]. In modulating multiple immune response cell types and their corresponding cytokines, EPO and its JM4 derivative transformed a highly uncontrolled autoimmune process into a more normalized condition while providing significant neuroprotective benefits in both clinical deficit and neuropathology.

Our small peptide EPO derivative JM4 was administered to acutely brain injured mice. JM4 offers a therapeutic window of at least 9 hours following injury. Results indicated remarkable reduction in neural cell death following TBI. JM4 shows promise as an effective tool in treating acute TBI and perhaps a broader range of acute neurological insults.

Authorship

BW was responsible for experimental design, animal studies, data acquisition, analysis, and interpretation, literature review, and preparation of the full manuscript. MK, MM, and ER were involved in data collection for most experiments performed in this work and the associated analysis and interpretation. MK also was involved in literature review and editing of the manuscript. WL was critical in performing several of the experiments including animal studies, and design of the EP-P peptides illustrated in the final figure of this work, as well as being responsible for revisions. XL was involved in data collection and analysis. YM was involved in experimental design and data interpretation as well as literature review and draft revisions. PD was responsible for oversight of the study, experimental design, data interpretation, and draft revisions. All authors have given final approval for the publication of this work and agree to be accountable for all aspects of this work.

Acknowledgements and Potential Conflicts of interest

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FIGURE LEGENDS

Fig. 1 Low magnification (2.0X) of cryostat sectioned brain lesions comparing JM4-treated TBI animal to sham-treated TBI control. The top panel shows the damaged areas following staining for hematoxylin and eosin. The middle panel shows fluorescent-labeled nuclear TUNEL staining for DNA fragmentation (TUNEL method with Cy3 detection). The lower panel shows higher magnifications (20X) of TUNEL stained lesions. The lesion in the JM4-treated animal shows markedly reduced cell death (TUNEL stain) and size compared to the PBS-treated control lesion at 3 days post-injury.

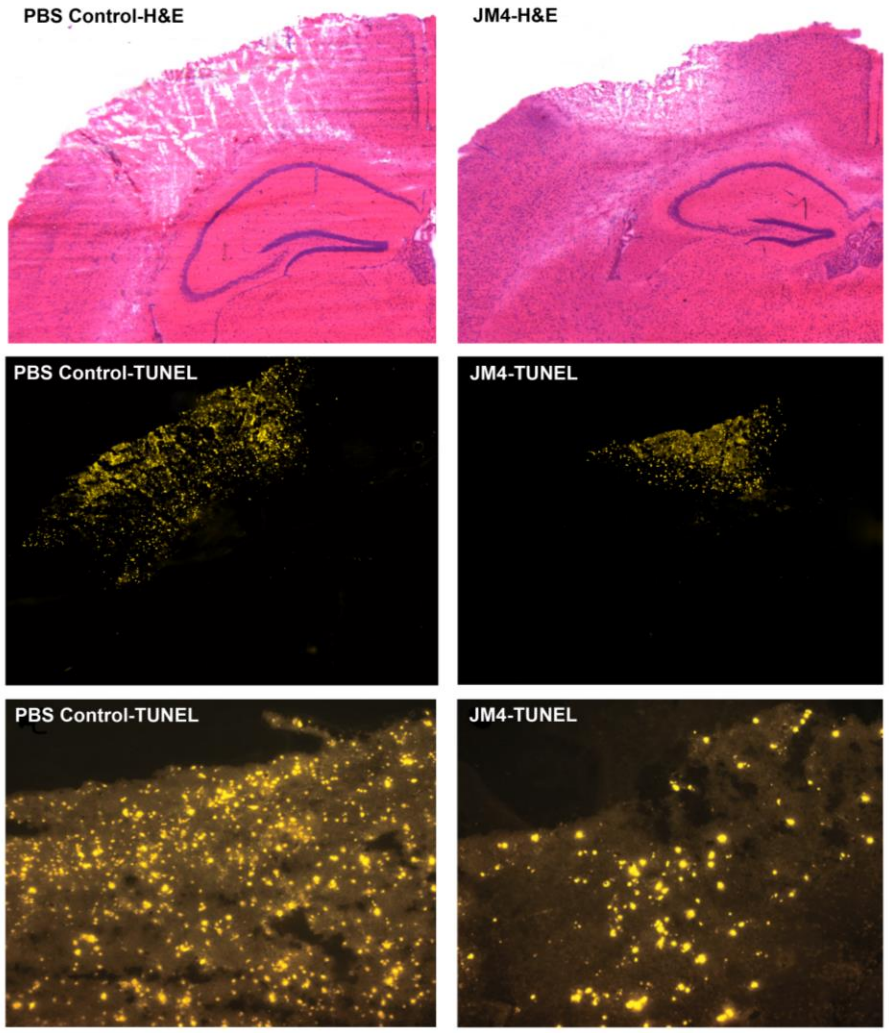


Fig. 2 Whole molecule EPO and its derivative peptide JM4 profoundly reduce cell death in the impacted hemisphere. (A) Treatment with EPO or JM4 significantly decreased TUNEL-positive cells in animals receiving 3 days of treatment compared to PBS sham-treated TBI animals (n = 10 for PBS and JM4, n = 11 for EPO, *** *P* <0.001). (B) A positive treatment effect was also seen in JM4-treated animals 8 days post-injury (n = 6 for each group, * *P*< 0.05).

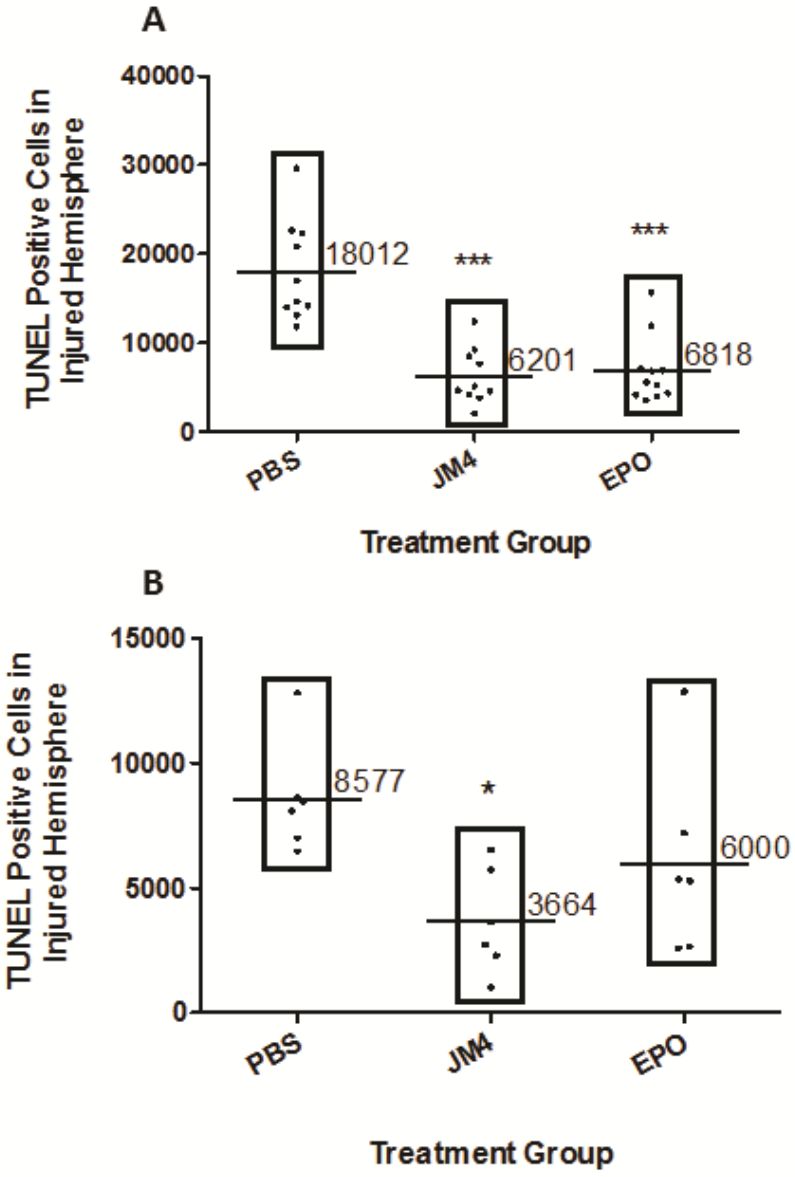


Fig. 3 Effect of JM4 therapy on the lesion area within the injured hemisphere (evaluated at 3 days and 8 days post-injury). For this experiment, a TUNEL-labeled slide containing sections spanning the entire hemisphere was used to quantify the total area containing dying cells. The JM4-treated group showed a marked reduction (50%) in lesioned area compared to PBS sham-treated animals at both (A) 3 days (**P <0.01, *** P <0.001) and (B) 8 days post-injury (*P <0.05).

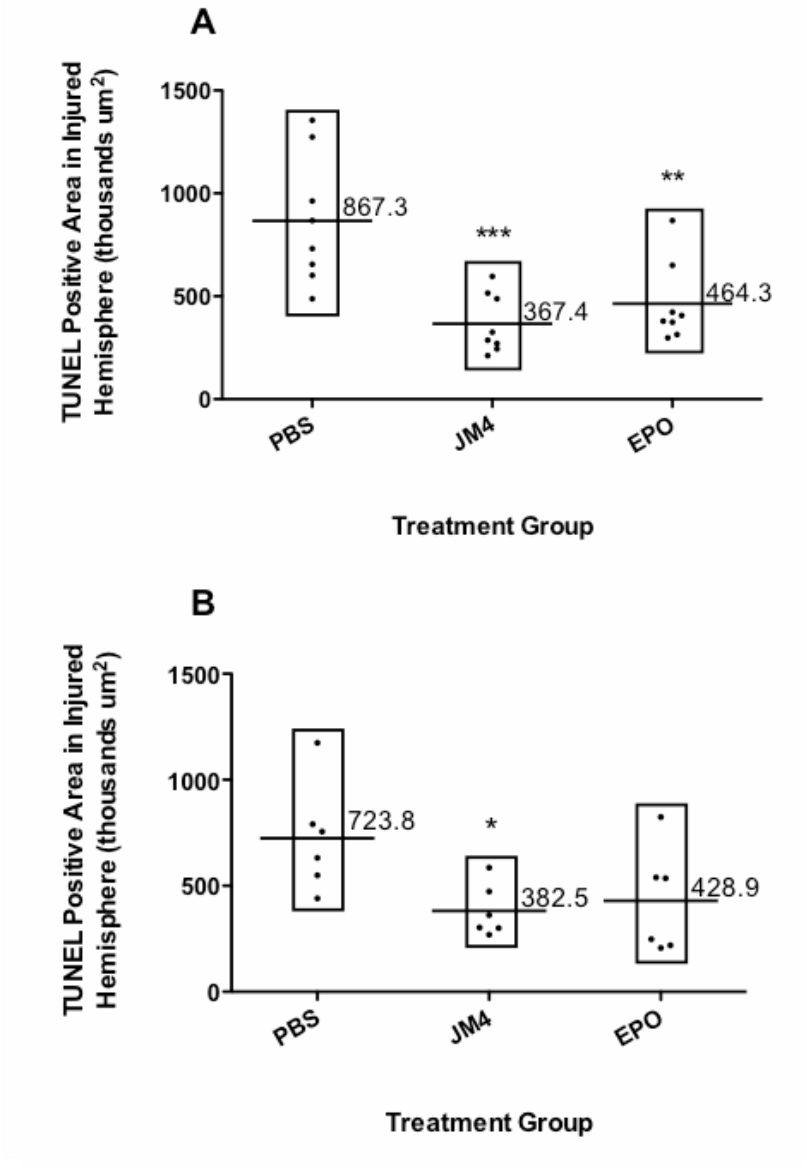


Fig. 4 A modified version of the Simple Neuroassessment for Asymmetric Impairment (SNAP) evaluation was used to determine if JM4 therapy has a treatment effect on neurologic deficit in brain injured animals. Evaluation was performed by blinded observers post-injury. JM4-treated TBI mice showed significant improvement in neurological deficit compared to PBS sham-treated TBI mice on a 29 point scale (score 0 indicates no deficit) following TBI. Significant positive effects were observed in this mixed design study particularly as time progressed when comparing PBS and JM4 treated animals (P <0.05). Data are represented as mean ± 95% CI.

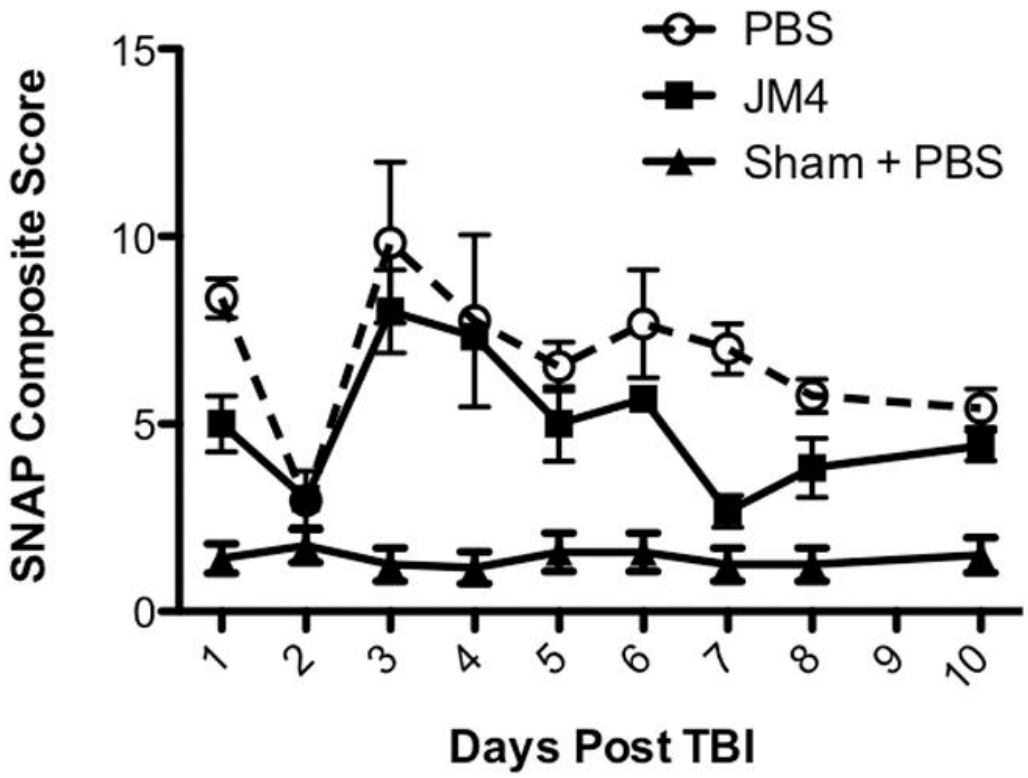


Fig. 5 Therapeutic window for JM4 therapy remains open for at least 9 hours post-injury. Mice were given identical cortical injuries and separated into groups. PBS sham-treated control TBI animals were injected with 200µl PBS 15 minutes post-TBI and 3 separate groups of TBI animals were administered an initial dose of JM4 (10 µg/mouse) at either 15 minutes, 3 hours, or 9 hours post-injury and subsequently treated once daily for 2 additional days before sacrifice. Cell death was quantified using the TUNEL reaction. Treatment groups given initial treatment at 15 minutes, 3 hours, or 9 hours post-injury all showed major reductions in TUNEL positive cell number compared to the sham-treated control group. The effectiveness of JM4 therapy, despite delayed initial administration, indicates that JM4's treatment window extends for at least 9 hours after TBI (**P* <0.01, ** *P* <0.001).

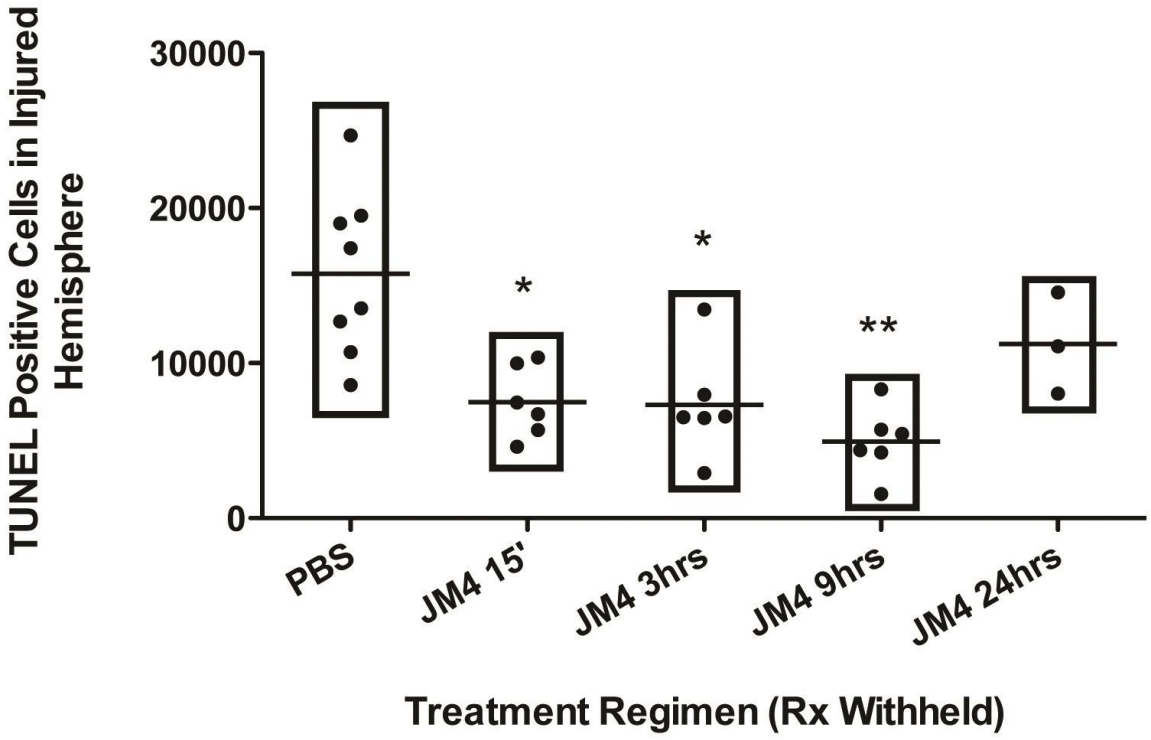
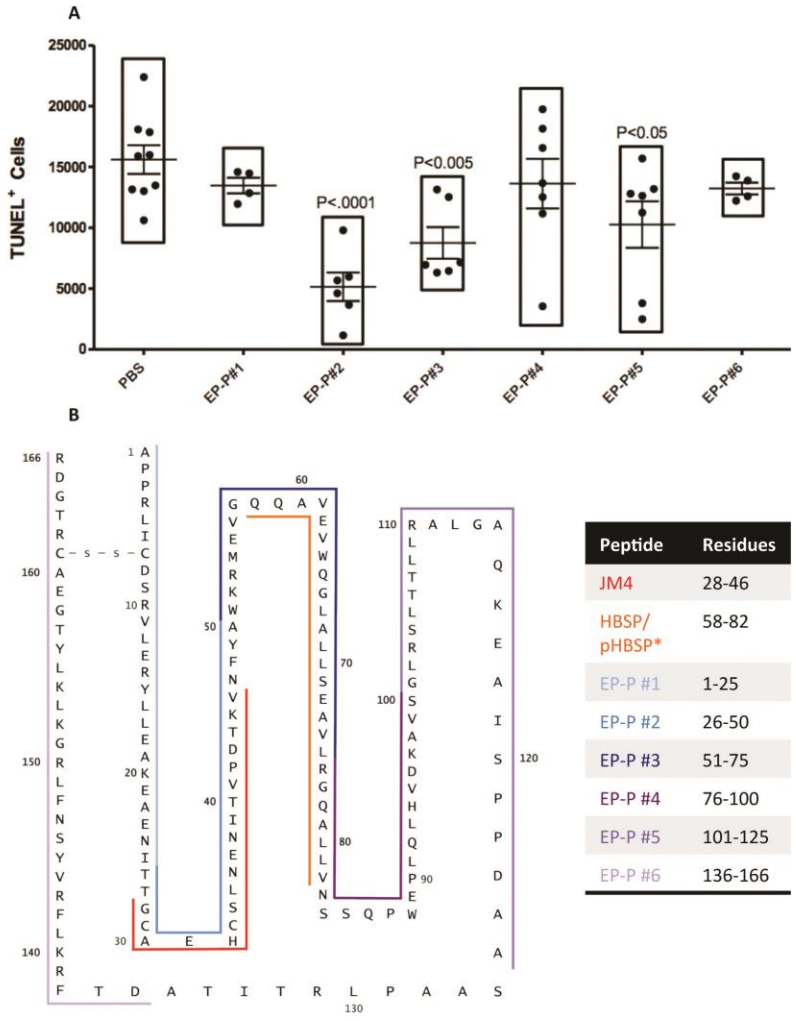


Fig. 6 The majority of the neuroprotection afforded by EPO is contained within a select early segment of the whole EPO molecule. (a) Treatment with EP-P#2 or EP-P#3 profoundly reduces cell death in the impacted hemisphere, significantly decreasing TUNEL-positive cells in animals receiving 3 days of treatment compared to PBS sham-treated TBI animals [n = 8 for each group, *P* <0.0001 (EP-P#2), *P* <0.005 (EP-P#3)]. (b) Map of whole EPO sequence, showing the positions of EP-P #1 - 6, JM4, and short peptides HBSP/pHBSP. The HBSP/pHBSP* in the figure key indicates that the molecules HBSP and pHBSP are not reflected by the primary structure of EPO and are rather derived from the aqueous face of three dimensional structure of helix B.



TABLES

Table 1. The brain/serum ratio of JM4 greatly exceeds that of inulin indicating JM4 likely crosses the BBB. The influx rate was 0.34±0.06µl/g-min, which is the typical value expected for a peptide the size of JM4 (~2kD) crossing the BBB by transcellular diffusion.

Brain/serum ratio	Normal #1	Normal #2	Normal #3
^{3}H-JM4	2.55	2.71	2.41
^{14}C-Inulin	0.35	0.99	1.22

SUPPLEMENTS

Supplement 1: The Simple Neuroassessment for Asymmetric Impairment (SNAP) procedure was modified in this study. The detailed guidelines depicted here were used to determine the composite SNAP score for each animal following TBI.

Score	0	1	2	3	4	5
A. Interactions	Avoids being handled; observer unable to grab skin on its back	Responds quickly only after briefly touched	Freezes before escaping; can be grabbed by skin on its back but sometimes scurries away	Responds after multiple nudges; able to be grabbed by skin on its back every time	Only moves head	Comatose
B. Cage Grasp	Both paws release simultaneously	CL paw first to release <50% of time. Ambiguous but is otherwise alert and active	CL paw first to release >50% but <100%	CL paw always first to release	CL paw does not grasp	Does not grasp with either paw
C. Visual Placing	Once aware of ledge, arches back and reaches out with both forepaws	May reach with both paws but IL leads. Twists body. Taps CL paw on ledge.	Occasionally does not reach with CL paw	Does not reach with CL paw at all	Head not raised and neither paw reaches	Comatose
D. Pacing or circling	Moves actively in random directions	Exhibits freezing, but able to be guided in either direction	Turns in one direction predominantly	Pacing, obstinate progression in same direction	Tight circling	Rolling
E. Gait/Posture and Head Tilt	Normal gait and posture and no head tilt	Somewhat off center but not immediately apparent	Body and/or head tilt clearly and immediately seen	Severe body and/or head tilt clearly and immediately seen	Unable to support weight on CL side	Recumbent
F. Baton 1. Speed	Normal	Mildly to moderately deficient	Almost totally deficient	Add baton scores together for a score of 0 to 4		
2. Accuracy	Normal	Mildly to moderately deficient	Almost totally deficient			

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