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Stem cell therapies for traumatic brain injury

“Inspired by some earlier success in models of ischemic brain injury, stem cell transplantation has shown some preclinical efficacy, primarily in models of focal traumatic brain injury.”

Keywords: contusion • diffuse axonal injury • induced pluripotent stem cells • mononuclear cells • neural stem cells • neuronal precursors • neurotrophins • oligodendrocyte precursors • regeneration • stroke

The clinical problem
Traumatic brain injury (TBI) is a common problem with unmet therapeutic needs. Although most cases of TBI are concussions that usually resolve over a few weeks, a great number of patients will suffer chronic disability from TBI-associated encephalopathies. Such conditions include focal contusions related to low-impact falls and diffuse axonal injury (DAI) from ultrafast loading of axons due to rotational acceleration in the course of motor vehicle crashes and other scenarios. Focal contusions are impact injuries featured by intraparenchymal hemorrhage with edema and ischemia in the inferior frontal and temporopolar regions leading to neuronal cell death and secondary axonal degeneration. DAI is an impulse injury associated with dynamic loading of axons and represents the commonest neuropathology across TBI causes and degrees of severity. Clinical problems caused by traumatic contusions and DAI have no satisfactory treatments besides symptomatic alleviation with physical/occupational/speech–language therapy and the empirical use of CNS-acting drugs. Clinical trials of small molecules have been unsuccessful [1]. Inspired by some earlier success in models of ischemic brain injury, stem cell transplantation has shown some preclinical efficacy, primarily in models of focal TBI. However, a number of limitations, including wide variance in transplanted cells and reported outcomes, make it difficult to draw general conclusions at this time.

Stem cells from bench to bedside
Recent discoveries of the ability of exogenous neural stem cells to become successfully incorporated into the neural parenchyma have refuted earlier notions on the mature nervous system as an environment unfavorable to ongoing developmental events [2,3]. Such neural stem cells are derived from fetal neural tissue, embryonic stem cells or somatic cells induced to pluripotency with specific transcription factors. Scaling up production is challenging for fetal cells. Embryonic stem cells and induced pluripotent stem cell sources are theoretically inexhaustible and extremely pliable and have been induced to a large number of neuronal or glial fates including motor [4] and dopaminergic [5] neurons as well as oligodendrocytes [6]. Work with human neural stem cells in animal models was instrumental in the initiation of pioneering clinical trials in motor neuron disease (NCT01348451, NCT01730716) and spinal cord injury (NCT01772810). Although it is too early to know the outcomes, these were landmark developments in regenerative neuroscience and are already followed by early trials in other disorders. The therapeutic effects of neural stem cells in these conditions are presumably due to a combination of synaptic physiological actions and the synaptically release of neuroprotective molecules [7]. In an interesting turn of events, regenerative medicine based on stem cells may look more and more like regenerative medicine based on neurotrophins and trophic cytokines in the 1990s, perhaps with the added benefit that...
stem cells can produce these molecules indefinitely (Figure 1).

**Preclinical modeling of TBI**

The last 20+ years have seen considerable efforts in modeling TBI by cause or mechanism. These models are usually classified into focal and diffuse. Focal models include the weight drop, controlled cortical impact injury and mid-line fluid percussion injury. Diffuse models are produced via inertial or impact acceleration. Modifications of fluid percussion injury have both focal and diffuse elements. These models have not been adequately tested in nude rats or SCID mice, in other words, subjects appropriate for stem cell transplantation, but this need has begun to be more recently addressed. Controlled cortical impact and impact acceleration models offer complementary opportunities for regenerative medicine: the former is a primary contusional injury with secondary axonal degeneration, whereas the latter is a model of DAI with secondary effects on neurons. Based on paradigms worked out in spinal cord injury, the previous models can be used to optimize neuronal-versus oligodendrocyte-based cell therapies: neuronal precursor transplants can be best optimized in models like controlled cortical impact, whereas oligodendrocyte precursor transplants can be best worked out in models of DAI. This contention does not imply that contusions are best treated with neuronal and DAI with oligodendrocyte transplants. As in the case of spinal cord injury, both neurons and oligodendrocytes may need to be replaced and a mixed transplantation approach would probably work best in clinical TBI scenarios [8].

**Stem cell transplantation as experimental therapy for TBI**

Because of the complexity of TBI, we need to identify specific targets for repair guided by pathological mechanisms. Such tasks may include replacing dead neurons or protecting injured axons and cell bodies and promoting axonal repair and regeneration (Figure 2). Neuronal degeneration or death is encountered in both focal injury and DAI. Focal TBI (contusion) and DAI present different challenges in this regard: contusions may respond to targeted transplantation but cell death is acute; transplantation in DAI may need to be multifocal, but perikaryal degeneration is slow and may or may not lead to cell death. Although axonal repair/remyelination as a therapeutic target is best established in spinal cord injury, demyelination may also contribute to axonal degeneration and disconnection within brain circuits.

A growing number of studies with systemically administered stem cells may disclose novel mechanisms of neural injury and repair. Cells typically used in this approach are derived from bone marrow and include mesenchymal, multipotent adult and mononuclear stem cells. Bone marrow-derived stem cells are easy to access, require simple or no manipulation and have no attached immune rejection concerns if they are patient derived. Mononuclear cells have already entered several clinical trials: these cells are relatively small and thence not trapped in the lungs (first-pass effect) after intravenous administration, whereas less than 4% of intravenously injected mesenchymal cells reach arterial circulation [10]. Bone marrow-derived cells have shown efficacy in stroke models. Because of questionable penetration into brain, one of the proposed therapeutic mechanisms is the modulation of immune response [10]. Consistent with this view, there is growing evidence for the role of spleen as an organ that modulates neural injury [11]. The presumed trophic effects of systemically delivered bone marrow cells require further clarification. One study that has shown good blood–brain barrier penetration in controlled cortical impact injury also found an increase in levels of neurotrophins and VEGF in brain [12], whereas other studies have shown poor penetration [13]. This discrepancy may be due to first-pass effects or the particulars of experimental models. At any rate, it is unclear how trophic effects can be induced if these cells do not cross the blood–brain barrier [14].

Although parenchymal or intravenous administration of stem cells in models of TBI and stroke is common, intrathecal or intraventricular delivery is also being used...
with some benefits. The potential of such strategies, especially the intraventricular route, for diffuse or multifocal effects may be greater compared with that of parenchymal strategies, especially if stem cells can be enticed to migrate to the lesion sites and differentiate into neural cells [15]. However, the consistency and comparative advantages of such effects are far from established.

The outcomes of preclinical testing of stem cells in TBI models have been recently reviewed by one of us [16]. Positive effects were observed in most studies with a small mean effect size that was more pronounced with modified or ‘enhanced’ cells. Transplantation within the lesion (for focal TBI) had a larger effect size than intravenous or ventricular delivery. Unfortunately, many of these studies have methodological problems. In addition, there is as yet no common standard for the assessment of outcome measures. Furthermore, a synthesis of studies using different stem cell preparations is extremely difficult. Also, the majority of TBI studies using human stem cell do not assess cell survival, thus clouding our understanding of potential mechanisms of action. Although transplanted stem cells such as neuronal precursors are fully capable of forming mature synapses with host structures in the brain and spinal cord [17], the physiological status of these synapses and their specific role in restoring function has not been characterized. Functionality of regenerated synapses is important not only for the purpose of conveying appropriate physiological signals but also for transsynaptic trophic support. The application of optogenetic strategies may prove critical in solving this problem [18].

**Special considerations for focal TBI & DAI**

Most published stem cell experiments in TBI are on focal models: about half of them used some form of controlled cortical impact, and the rest are equally split between weight drop and fluid percussion. For reasons explained in the previous paragraph, the field is behind stroke and spinal cord injury. Although a variety of benefits have been reported, integration with the host has not been demonstrated. Also unresolved are the questions of optimal dosing and dose scaling to man; this is a tricky issue because, at least based on our experience on spinal cord injury, dose escalation alters the dynamics of engraftment, migration and fate [19]. Even less can be said about stem cell therapies for models of DAI, although the field can borrow from spinal cord injury that invariably involves trauma in axonal tracts. In contrast to spinal cord injury, where axons course in restricted areas, DAI involves disparate axon tracts that would be difficult to transplant at the same time. Therefore, in the case of DAI, the choice of transplantation route (systemic, ventricular or parenchymal) and location of transplant (if we select parenchymal delivery) are critical. A recent study has shown that, in sharp contrast to neuronal progenitors, human oligodendrocyte progenitors do not colonize and differentiate locally but rather migrate massively along white matter tracts and remain within the white matter, often ensheathing themselves around host axons [20].

**Figure 2. Sketch of repair targets for stem-cell based therapies in traumatic brain injury.** In upper panel, neuronal precursors support injured neurons (1) or, after they differentiate into nerve cells, they replace dead neurons (2). In lower panel, transplanted oligodendrocyte precursors support injured axons and prevent their degeneration (1) or facilitate the growth and maturation of axon sprouts (2). Immune signals related to systemically delivered bone marrow cells are not included.

**Conclusion**

After multiple failures in clinical trials of single and combination agents, TBI is in dire need for effective treatments. The nature of some of the key lesions invites the consideration of the toolkit of regenerative medicine, including stem cell transplants. Important advancements in preclinical stem cell therapeutics and the popularity of TBI models create unprecedented opportunities for discoveries that could push this stalled field forward. Although there is no lack of interesting data, a great disparity in models, cell preparations, and reported outcomes detract from an enthusiastic endorsement of stem-cell therapeutics for TBI at this time. Consortia to establish guidelines for TBI modeling and NIH initiatives supporting collaborative and replication platforms are urgently needed: it makes no sense to only fund original research that would make little difference in and of itself and would be difficult to replicate or integrate with other studies. In the course of experimenting with stem cell therapeutics, one of
the greatest promises is the discovery of physiological molecular signals that afford protection or promote recovery in the adult brain.

Financial & competing interests disclosure
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No writing assistance was utilized in the production of this manuscript.

References

Editorial Koliatsos, Xu & Cummings
Progenitor cell therapy for traumatic brain injury: effect of serum osmolarity on cell viability and cytokine production

**Introduction:** The potential translation of mesenchymal stem cell (MSC) therapy into a multimodal protocol for traumatic brain injury requires evaluation of viability and cytokine production in a hyperosmolar environment. Optimization of MSC therapy requires delivery to the target area without significant loss of cellular function or viability. No model evaluating the potential efficacy of MSC therapy at varying osmolarities currently exists. **Methods:** Rat MSCs were characterized with flow cytometric immunophenotyping. MSCs (passage 3) were placed in culture with multipotent adult progenitor cell media at varying osmolarities (250, 270, 290, 310, 330, 350 and 370 mOsm) potentially found with hypertonic saline infusion. After culture for 24 h, cellular viability was measured using flow cytometry (n = 6). Next, brain tissue supernatant was harvested from both normal rat brains and injured brains 6 h after cortical injury. Subsequently, MSCs were placed in culture with multipotent adult progenitor cell media ± 20% normal brain or injured brain supernatant (at the aforementioned osmolarities) and allowed to remain in culture for 24 h (n = 11). At this point, media supernatant cytokine levels were measured using a multiplex cytokine assay system. **Results:** MSCs showed no clinically significant difference in viability at 24 h. MSCs cultured with 20% injured brain supernatant showed an decrease in proinflammatory cytokine production (IL-1α and IL-1β) with increasing osmolarity. No difference in anti-inflammatory cytokine production (IL-4 and IL-10) was observed. **Conclusion:** Progenitor cell therapy for traumatic brain injury may require survival and activity in a hyperosmolar environment. Culture of MSCs in such conditions shows no clinically significant effect on cell viability. In addition, MSC efficacy could potentially be enhanced via a decrease in proinflammatory cytokine production. Overall, a multimodal traumatic brain injury treatment protocol based upon MSC infusion and hypertonic saline therapy would not negatively affect progenitor cell efficacy and could be considered for multicenter clinical trials.

**KEYWORDS:** cytokines, inflammation, mesenchymal stem cells, osmolarity

In the USA, 1.5 million patients suffer a traumatic brain injury (TBI) each year, resulting in 50,000 deaths with an additional 230,000 patients requiring hospitalization [1]. The overall prevalence of TBI is estimated to be 6.5 million people [2]. Of those patients affected, up to 48% are impaired by physical, cognitive and psychosocial deficits [3]. Beginning aggressive rehabilitation early in the patient’s hospital course has been shown to lead to improvement in functional status [4,5]; however, neurons show little ability to repair and no treatment modality is currently available to reverse acute brain injury. A large body of work has failed to show significant efficacy from single-agent pharmacologic neuroprotective therapies. Therefore, the NIH recently convened a meeting to discuss the complex pathophysiology of neuronal injury and the failure of all trials based on current mono-therapies (controlled hypothermia, hyperosmotic infusion) to date. Recommendations included the development of in vitro models to research multimodality treatments that could target several mechanisms of TBI’s complex pathophysiology [6,10]. Two therapeutic modalities currently under investigation for the treatment of TBI are the infusion of hypertonic saline (HTS) and progenitor cell therapeutics.

Preliminary research has shown that HTS infusion after TBI could offer potential neuroprotection. One possible pathway could be via a decrease in intracranial pressure (ICP). Both preclinical research using a dog model [7] and prospective randomized trials [8] have shown decreased ICP after HTS infusion without significant effect on cerebral blood flow (CBF). Additional investigation completed by Khanna et al. demonstrated that HTS infusion in a pediatric population was associated with improved CBF in accordance with a decrease in ICP [9]. HTS resuscitation also increases mean arterial pressure, thereby attenuating a significant increase in poor neurological outcomes seen with hypotension within 6 h of TBI [10]. Furthermore,
Violet et al. have shown HTS infusion to be more effective than mannitol as a hyperosmolar agent after TBI [11]. Despite promising initial results, the effect of HTS infusion upon ICP remains controversial as alternate prospective trials have failed to show improvement in ICP control when compared with crystalloid (lactated ringer’s) infusion [12,13]. HTS infusion decreased the number of complications, the number of required interventions and length of intensive care unit stay after TBI in a pediatric population [14]; however, additional studies completed with an adult population failed to show a favorable effect on the required interventions [13]. Despite the promising results noted in the acute and subacute setting, some trials have failed to show sustained improved neurological outcomes 6 months after TBI [6,15]. As a result of the controversial results derived from the initial preclinical and clinical trials, the Joint Section on Neurotrauma and Critical Care did not make any recommendations for the use HTS infusion for TBI [16]; however, the potential neuroprotection observed specifically in the pediatric population requires additional investigation.

A large amount of research has been completed to investigate the potential role of progenitor (stem) cell therapeutics for the treatment of TBI. While initial preclinical research has shown a potential benefit from progenitor cell therapeutics [17–20], the precise mechanism remains intensely controversial. Furthermore, the secondary injury observed with TBI is associated with an induction of the systemic inflammatory response. Analysis of rat brain supernatant from a TBI model has shown significant increase in the proinflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α in both the direct injury and penumbral areas [21]. In addition, previous preclinical work has shown progenitor cells to migrate towards the site of injury and potentially modulate cytokine production. Co-culture of mesenchymal stem cells (MSCs) with purified immune cells has shown a decrease in proinflammatory cytokine production (TNF-α and IFN-γ) with an increase in production of the anti-inflammatory cytokines IL-4 and IL-10 [22]. Therefore, MSCs could offer neuroprotection via modulation of locoregional proinflammatory cytokine production in accordance with the production of anti-inflammatory cytokines.

Previous research has shown HTS infusion and progenitor cell therapeutics to be ideal potential candidates for multimodal TBI treatment regimens. The potential translation of MSC therapy into such a multimodal protocol requires evaluation of cell viability and cytokine production in a hyperosmolar environment. Previous research has shown osmotic stress to induce apoptotic cell death in multiple cell lines including mononuclear cells [23], cardiomyocytes [24] and fibroblasts [25]. Furthermore, hyperosmotic stress has been shown to increase proinflammatory cytokine production from human peripheral blood mononuclear cells [26]. No model evaluating the potential efficacy of MSC therapy at varying osmolarities currently exists. We designed a series of in vitro experiments to investigate MSC viability and cytokine production while cultured at the osmolarities observed with HTS infusion for the treatment of TBI.

Experimental design
Mesenchymal stem cells (passage 3) were placed in culture with multipotent adult progenitor cell (MAPC) media with the varying osmolarities (250, 270, 290, 310, 330, 350 and 370 mOsm) potentially found with HTS infusion. After 24 h in culture, cellular viability was measured using flow cytometry (n = 6 samples per media osmolarity). Next, brain tissue supernatant was harvested from both normal rat brains and injured brains 6 h after cortical injury. Subsequently, MSCs were placed in culture with MAPC media ± 20% normal brain or injured brain supernatant (at the aforementioned osmolarities) and allowed to remain in culture for 24 h (n = 11 samples per media osmolarity). At this point, media supernatant cytokine levels were measured using a Bio-Plex cytokine assay system (Bio-Rad Laboratories, Hercules, CA, USA).

Ethical approval
All protocols involving the use of animals were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Texas Institutional Animal Care and Use Committee (protocol HSC-AWC-07–055).

Data analysis
Unless otherwise indicated, all values are represented as mean ± standard error of the mean. Values were compared using analysis of variance (ANOVA) with a post-hoc Dunnett analysis using an osmolarity of 290 as the control. A p-value of ≤0.05 was used to denote statistical significance.

Isolation, characterization & labeling of rat MSCs
Mesenchymal stem cells were isolated from the bone marrow of Sprague-Dawley rats and expanded in MAPC media as previously...
described [27]. Flow cytometric immunophenotyping was used to ensure that the MSCs were CD11b−, CD45−, CD29+, CD49e+, CD73+, CD90+, CD105+ and Stro-1+. Passage 3 cells were used for all experiments.

**Measurement of media osmolarity**
The osmolarity of MAPC media was measured using the 5500 vapor pressure osmometer (Wescor Inc., Logan, UT, USA). Adjustments in media osmolarity were completed by addition of sterile distilled water or sodium chloride.

**MSC viability**
Mesenchymal stem cells were removed from the culture plates and incubated with propidium iodide for 10 min. Using flow cytometry, the fraction of dead cells was calculated.

**Controlled cortical impact injury**
A controlled cortical impact (CCI) device (eCCI Model 6.3; VCU, Richmond, VA, USA) was used to administer a unilateral brain injury as described previously [28]. Male Sprague Dawley rats weighing 225–250 g were anesthetized with 4% isoflurane and a 1:1 mixture of N₂/O₂ and the head was mounted in a stereotactic frame. With the head held in a horizontal plane, a midline incision and subsequent 7- to 8-mm craniectomy was performed on the right cranial vault. The center of the craniectomy was placed at the midpoint between bregma and lambda, 3 mm lateral to the midline and overlying the tempoparietal cortex. Animals received a single impact of 3.1-mm depth of deformation with an impact velocity of 5.8 m/s and a dwell time of 150 ms (moderate-to-severe injury) at an angle of 10° from the vertical plane using a 6-mm diameter impactor tip, making the impact orthogonal to the surface of the cortex. The impact was delivered onto the parietal association cortex. Sham injuries were performed by anesthetizing the animals, making the midline incision, and separating the skin, connective tissue and aponeurosis from the cranium. The incision was then closed. The body temperature was maintained at 37°C by the use of a heating pad. Previously obtained serial arterial PaO₂ and PaCO₂ measurements have shown that animals do not become hypoxic or hypercarbic during this procedure [21].

**Brain homogenate supernatant fluid collection**
Rats were sacrificed 6 h after CCI or sham injury. Their brains were extracted and four regions, relative to the injury, were isolated: the site of direct injury, penumbral region, ipsilateral frontal region and contralateral region, as we have previously described [21]. The sections were weighed to ensure each section was 120 mg, gently minced with a pellet pestle, diluted in 1 ml (low-glucose Dulbecco’s modified Eagle medium with 10% fetal bovine serum; Gibco, Carlsbad, CA, USA), vortexed for 30 s and centrifuged for 6 min at 1000 g. The supernatant, containing intracerebral, interstitial fluid, was collected [21]. Of note, only the supernatant harvested from the direct injury region was used for stimulation of MSC cultures.

**Cytokine analysis**
Cytokines were detected in the MSC media supernatant using the Bio-Plex cytokine assay system (Bio-Rad Laboratories, Hercules, CA, USA). Concentrations of IL-1α, IL-1β, IL-4, IL-6, IL-10 and TNF-α were simultaneously evaluated using a commercially available multiplex bead-based immunoassay (Rat 9-Plex; Bio-Rad Laboratories). The assay was performed per the manufacturer’s instructions and the details have been previously published by our group and others [29,30]. High standard curves (low RP1 target value) for each soluble cytokine were used, ranging from 2 to 32,000 pg/ml. A minimum of 100 beads per cytokine region were evaluated and recorded. Values with a coefficient of variation beyond 10% were not included in the final data analysis. All samples were run in duplicate.

*Statistical significance (one-way ANOVA with Dunnett’s post-hoc) compared with control osmolarity (290 mOsm) (p < 0.05).
Results
Mesenchymal stem cells placed in culture with MAPC media at the various potential osmolarities observed with HTS therapy (250–370 mOsm) showed a narrow range of viability (91.4–93.9%). Statistical analysis using an osmolarity of 290 to represent a physiologic control showed difference for osmolarities of 250, 330, 350 and 370. Figure 1 outlines MSC viability at the varying osmolarities.

Table 1. Proinflammatory cytokine concentration (pg/ml) measured in the media (with and without 20% normal/injured brain supernatant) of mesenchymal stem cell cultures at varying osmolarities.

<table>
<thead>
<tr>
<th></th>
<th>250 mOsm</th>
<th>270 mOsm</th>
<th>290 mOsm*</th>
<th>310 mOsm</th>
<th>330 mOsm</th>
<th>350 mOsm</th>
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<tbody>
<tr>
<td><strong>IL-1α</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Media</td>
<td>18.5 ± 0.4</td>
<td>17.6 ± 1.0</td>
<td>18.0 ± 0.5</td>
<td>21.0 ± 1.6</td>
<td>19.3 ± 1.9</td>
<td>20.6 ± 1.6</td>
<td>19.1 ± 1.2</td>
</tr>
<tr>
<td>Normal brain supernatant</td>
<td>26.0 ± 0.4</td>
<td>30.1 ± 1.2</td>
<td>25.7 ± 0.8</td>
<td>33.2 ± 3.8</td>
<td>35.1 ± 3.2</td>
<td>38.1 ± 4.1</td>
<td>38.3 ± 6.2</td>
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<tr>
<td>Injured brain supernatant</td>
<td>399 ± 13.3</td>
<td>414 ± 10.6</td>
<td>448 ± 6.7</td>
<td>463 ± 31.3</td>
<td>452 ± 18.2</td>
<td>422 ± 10.3</td>
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**IL-1β**

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<th>290 mOsm*</th>
<th>310 mOsm</th>
<th>330 mOsm</th>
<th>350 mOsm</th>
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<tr>
<td>Media</td>
<td>18.5 ± 0.4</td>
<td>17.6 ± 1.0</td>
<td>18.0 ± 0.5</td>
<td>21.0 ± 1.6</td>
<td>19.3 ± 1.9</td>
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<tr>
<td>Normal brain supernatant</td>
<td>33.3 ± 2.4</td>
<td>34.9 ± 2.6*</td>
<td>32.4 ± 1.3</td>
<td>38.9 ± 2.8</td>
<td>39.0 ± 2.2</td>
<td>39.8 ± 2.9</td>
<td>43.4 ± 4.1*</td>
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<tr>
<td>Injured brain supernatant</td>
<td>100 ± 3.7</td>
<td>92 ± 3.1</td>
<td>110 ± 6.5</td>
<td>108 ± 2.3</td>
<td>102 ± 3.7</td>
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**IL-6**

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<th>290 mOsm*</th>
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<th>330 mOsm</th>
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<tr>
<td>Normal brain supernatant</td>
<td>123 ± 33</td>
<td>131 ± 37</td>
<td>108 ± 28</td>
<td>110 ± 28</td>
<td>112 ± 29</td>
<td>123 ± 25</td>
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<td>Injured brain supernatant</td>
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<td>161 ± 42</td>
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**TNF-α**

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<th>330 mOsm</th>
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<td>Injured brain supernatant</td>
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<td>10.6 ± 0.6</td>
<td>10.9 ± 0.9</td>
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<td>9.9 ± 0.6</td>
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*Statistical significance (one-way ANOVA with Dunnett’s post-hoc) compared with control osmolarity (290 mOsm) (p < 0.05).

Table 2. Anti-inflammatory cytokine concentration (pg/ml) measured in the media (with and without 20% normal/injured brain supernatant) of mesenchymal stem cell cultures at varying osmolarities.

<table>
<thead>
<tr>
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<th>250 mOsm</th>
<th>270 mOsm</th>
<th>290 mOsm*</th>
<th>310 mOsm</th>
<th>330 mOsm</th>
<th>350 mOsm</th>
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<tbody>
<tr>
<td><strong>IL-4</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>110 ± 3.6</td>
<td>103 ± 5.8</td>
<td>103 ± 2.2</td>
<td>107 ± 9.3</td>
<td>95 ± 7.8</td>
<td>119 ± 8.4</td>
<td>129 ± 9.1</td>
</tr>
<tr>
<td>Normal brain supernatant</td>
<td>213 ± 11.2</td>
<td>234 ± 10.4</td>
<td>207 ± 6.7</td>
<td>243 ± 16.6</td>
<td>222 ± 21.1</td>
<td>242 ± 17.6</td>
<td>252 ± 16.9</td>
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<tr>
<td>Injured brain supernatant</td>
<td>213 ± 34.7</td>
<td>220 ± 36.7</td>
<td>220 ± 39.3</td>
<td>204 ± 28.3</td>
<td>199 ± 23.5</td>
<td>184 ± 37.2</td>
<td>201 ± 35.6</td>
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**IL-10**

<table>
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<tr>
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<th>290 mOsm*</th>
<th>310 mOsm</th>
<th>330 mOsm</th>
<th>350 mOsm</th>
<th>370 mOsm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>2.6 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>2.0 ± 0.0</td>
<td>2.6 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Normal brain supernatant</td>
<td>3.5 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>3.3 ± 0.3</td>
<td>3.1 ± 0.4</td>
<td>3.4 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>Injured brain supernatant</td>
<td>3.7 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td>4.4 ± 0.4</td>
<td>3.7 ± 0.3</td>
<td>4.0 ± 0.3</td>
<td>3.6 ± 0.4</td>
<td>3.5 ± 0.2</td>
</tr>
</tbody>
</table>

*Statistical significance (one-way ANOVA with Dunnett’s post-hoc) compared with control osmolarity (290 mOsm) (p < 0.05).
an osmolarity of 370 for MSCs cultured with injured brain supernatant (compared with the control value of 290). In addition, for MSCs cultured with injured brain supernatant there was a decrease in IL-1β production at serum osmolarities of 270 and 370 (p = 0.02) (Table 1). Further analysis failed to show a difference in the production of TNF-α or IL-6 when cultured in injured brain supernatant. In addition, no difference was observed for MSCs cultured in media alone or with 20% normal brain supernatant.

Table 2 shows anti-inflammatory cytokine production (IL-4 and IL-10) measured in the media (with and without 20% normal/injured brain supernatants) of MSC cultures after 24 h. No difference was observed for IL-4 or IL-10 at any osmolarity.

Discussion
Multimodal protocols for the treatment of TBI could require the intravenous infusion of MSCs with HTS therapy. Optimal cell efficacy requires adequate viability and function at elevated serum osmolarities. Our data show that MSC viability (91.4–93.9%) is largely unaffected by the range of serum osmolarities (250–370 mOsm) that could be observed in a traumatically injured patient. While a significant increase in MSC viability is observed with increasing osmolarity, difference in viability fails to reach clinical significance.

The potential neuroprotection observed with HTS therapy after TBI could be derived from a decrease in proinflammatory cytokine production. Recent investigation has shown an increase in plasma matrix metalloproteinases (MMPs) as well as IL-6 after TBI in both animal models and injured patients [31,32]. MMPs are believed to contribute to further ischemic brain injury via breakdown of the blood–brain barrier. While initial work has shown controlled hypothermia to decrease the levels of MMP after TBI [32,33], little investigation into the effect of HTS has been completed. However, HTS therapy has been shown to mediate hepatic MMP production in a pancreatitis model and could offer neuroprotection via a similar pathway after TBI [34].

The observed benefit from MSC therapy for TBI could be explained by modulation of the locoregional or systemic inflammatory milieu [17]. While still controversial, the implanted MSCs could be responsible for the production in anti-inflammatory cytokines that lead to enhanced neuroprotection. Our data show a decrease in production of the proinflammatory cytokines IL-1α and IL-1β with increasing osmolarity. In addition, the production of the anti-inflammatory cytokines IL-4 and IL-10 remains unaffected with an increase in osmolarity. These data show that increasing osmolarity does not affect MSC function in the form of anti-inflammatory cytokine production. In addition, increasing osmolarity may be of some benefit as evidenced by a decrease in the pro-inflammatory cytokine concentration, which could lead to enhanced neuroprotection.

Conclusion
Traumatic brain injury is a major burden on the healthcare system worldwide. Current therapy in the acute setting is supportive with no pharmacologic treatment to attenuate neuronal cell death available today. The development of multimodal treatment protocols to attack TBI’s complex pathophysiology at multiple points could offer the most effective form of neuroprotection. The combination of HTS infusion and progenitor cell therapies could lead to improved CBF as well as modulation of the inflammatory response leading to reduced neuronal death. Our data have shown that the intravenous infusion of MSCs into the hyperosmolar environment seen with HTS therapy has no clinically significant effect on cell viability. In addition, MSC efficacy could potentially be enhanced via a decrease in pro-inflammatory cytokine production. Overall, a multimodal TBI treatment protocol based upon MSC infusion and HTS therapy would not negatively affect progenitor cell efficacy and could be considered for multicenter clinical trials.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.
Executive summary

**Traumatic brain injury**
- Traumatic brain injury affects 1.5 million people per year in the USA. Of those affected, up to 48% have chronic physical, cognitive or psychosocial deficits. No single modality pharmacologic treatment has shown efficacy.

**Hypertonic saline therapy**
- Preliminary trials have shown potential neuroprotection via control of intracranial pressure with improvement of cerebral blood flow; however, trials have failed to show cognitive improvement 6 months after injury.

**Progenitor cell therapy**
- Initial in vivo models have shown potential neuroprotection based upon intravenous delivery; however, a review of the literature shows no evidence of mesenchymal stem cell (MSC) viability at hyperosmolar conditions. Owing to NIH recommendations to develop multimodality therapeutic regimens for traumatic brain injury, a model to look at MSC viability and function is needed.

**MSC viability**
- MSCs showed a decrease in IL-1α and -1β production with increasing osmolarity. No difference in anti-inflammatory cytokine production was observed.
- Culture of MSCs at hyperosmolar conditions shows no clinically significant effect on cell viability. Additionally, MSC efficacy could potentially be enhanced via a decrease in pro-inflammatory cytokine production.

**Inflammatory cytokine production**
- MSCs cultured at various osmolarities (250–370) showed no clinically significant difference in viability (91.4–93.9%).

### Bibliography


Website

Progestosterone treatment for brain injury: an update

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Traumatic brain injury is a significant clinical problem for which there is still no effective treatment. Recent laboratory and clinical data demonstrate a potentially beneficial role for neurosteroids, such as progesterone and allopregnanolone, in the treatment of traumatic brain injury, ischemic stroke and some neurodegenerative disorders. Unlike single-target agents, progesterone affects many of the molecular and physiological processes in the cascade of secondary damage after a traumatic brain injury. This article updates a 2006 Future Neurology review of the research on progesterone and its metabolites in the treatment of traumatic brain injury, and presents new evidence that vitamin D deficiency can reduce progesterone neuroprotection, while combining progesterone with vitamin D produces better functional outcomes after TBI compared with either treatment alone.

Background
When our review of the role of progesterone (PROG) in brain injury was published in Future Neurology in 2006, there was no clinically available treatment for traumatic brain injury (TBI) [1]. This is still the case today. TBI is a major clinical concern in the USA and worldwide, and has been receiving increased public attention (and more funding) partly owing to the large number of concussive blast injuries suffered in the conflicts in Iraq and Afghanistan. US government sources indicate that, from 2003 to 2007, as many as 43,779 surviving combat casualties have been diagnosed with varying degrees of brain injury caused by explosive devices [101]. Taken together with more than 1.5 million annual cases of TBI occurring in the USA alone (including 300,000 annual sports-related injuries in children and young adults) [102], it is evident that brain injury continues to represent a substantial clinical and social problem.

Despite these grim data, there are grounds for optimism. Two independent Phase II clinical trials have now reported that PROG can be administered with a delay of up to 6 h or more after injury and still show beneficial results. The Progesterone for Traumatic Brain Injury, Experimental Clinical Treatment (ProTECT) trial was a randomized, double-blind, placebo-controlled trial with 100 patients suffering from moderate-to-severe brain injury (Glasgow Coma Scale [GCS] scores of 4–12) [2]. The patients treated with PROG were started on an intravenous (iv.) drip of 0.71 mg/kg at 14ml/h for the first hour, reduced to 0.50 mg/kg at 10 ml/h for the next 11 h. Five additional 10-h infusions at 10 ml/h were administered for the remainder of the 3 days of treatment. No serious adverse events were noted, and patients with severe injuries receiving 3 days of iv. PROG starting within 6–8 h after injury demonstrated a more than 50% reduction in mortality at 30 days compared with controls. The group with moderate injuries also showed significant ‘encouraging signs of improvement’ on their Disability Rating Scale (DRS) outcomes at 30 days compared with patients receiving placebo. The researchers concluded that PROG was beneficial for patients with both severe and moderate injuries, although the results were somewhat confounded in the severely injured by the fact that many in the group administered PROG survived who would not have otherwise survived without the treatment.

The ProTECT trial results were supported by another single-center trial of 159 severely brain-injured subjects (GCS score ≤ 8) [3] that tracked patient outcomes for a longer period. The 82 subjects in the PROG group were treated for 5 days with intramuscular injections of PROG (1.0 mg/kg intramuscularly every 12 h for a total of 5 days) started within 8 h of injury and demonstrated significantly better survival as well as functional outcomes at both 3 and 6 months compared with the 77 patients given placebo. A dichotomized analysis demonstrated that at the 3-month follow-up, 47% of the PROG-treated patients showed better functional outcomes on the GCS compared with 31% of the placebo group. At the 6-month follow-up the results were similar, with favorable outcomes in 58% of the PROG group compared with 42% of the placebo group. The PROG group had 18% mortality at 6 months, while the placebo group had 32% mortality at 6 months. It is important to emphasize that in both clinical trials PROG not only reduced mortality, but also significantly improved functional

Keywords
functional recovery, neurosteroids, progesterone, traumatic brain injury
outcomes. Although these reports need to be confirmed in larger multicenter trials, these two trials are the first pharmacological intervention for TBI to show a substantial benefit in human patients [4].

Based on these promising findings, the NIH is funding a national Phase III multicenter trial to test PROG in over 1000 moderately to severely brain-injured patients. This trial, termed ProTECT III, is designed to determine the efficacy of administering IV. PROG (initiated within 4 h of injury and administered for 72 h, followed by an additional 24-h taper) versus placebo for treating victims of moderate-to-severe acute TBI (GCS score of 12–4). The trial will track mortality, number of adverse and serious adverse events, DRS, and cognitive, neurological and functional outcomes.

Since the publication of the successful Phase II trials in 2007–2008 [2,3], there has been more interest in determining the extent to which PROG and some of its metabolites can enhance neuroprotection after different kinds of CNS injury. From just a few studies beginning in the 1990s, there are now over 100 published preclinical studies from 25 different laboratories, using four different species in 22 different injury models, demonstrating that PROG and its metabolites may be highly neuroprotective. Many of these studies have been published within the last few years.

Here we summarize recent findings illuminating some of the neuroprotective mechanisms of PROG and some of its metabolites. Further details can be found in extensive reviews by Brinton et al. [5], Schumacher et al. [6], Singh et al. [7], Stein and Hurn [8] and Stein [9]. The earlier literature on PROG focused mainly on the phenomenological markers of CNS repair so that, for example, if administered after a TBI, the hormone was shown to reduce cerebral edema, re-establish the compromised BBB, improve vascular tone, downregulate the expression of inflammatory factors, reduce excitotoxic damage and prevent post-traumatic seizures [5–10]. As these positive studies began to accumulate, attention turned to the question of how PROG was exerting its effects at the molecular level (whether this occurred through the classical PROG receptor or through other pathways).

**Update on progesterone & recovery from brain injury: mechanisms & systems**

**Overview**

Over two decades ago it was first noted that female rats show better recovery of cognitive function than their male counterparts after bilateral damage to the medial frontal cortex (MFC). This effect was then found to vary with hormonal cycles and was due to endogenous levels of PROG, which in turn suggested the possibility of using exogenously administered PROG in order to improve recovery in both males and females [11]. Since then, the effectiveness of PROG treatment in functional recovery after TBI has been repeatedly demonstrated [5,6,9,12,13], with most of the work focusing on the mechanisms of action. We now believe that the neuroprotective efficacy of PROG is a result of its actions on multiple genomic, proteomic and receptor systems, rather than on a single mechanism. It has now also become more clearly established that moderate and severe TBI cause systemic injuries that alter metabolic and physiological functions throughout the body [14]; an effective treatment should therefore have systemic effects.

**Molecular effects of progesterone**

A significant problem with damage to the CNS is disruption of blood flow to the local area of injury, resulting in loss of oxygen and glucose, energy failure and, ultimately, cell death. PROG has been demonstrated to protect neurons from ischemia and to decrease the size of the damaged area [8,15]. This resistance to injury is probably owing to several protective mechanisms, including maintenance of mitochondrial function, increased prosurvival signaling and reduced proapoptotic signaling [6,16–21].

PROG affects mitochondria in multiple ways. It helps restore them to normal morphology even after they have undergone severe vacuolation [22], inhibits the release of proapoptotic cytochrome c [23,24], and reduces the levels of proapoptotic B-cell lymphoma (Bcl)-2-associated X protein (Bax), Bcl-2-associated death-promoter (BAD) and activated caspase-3 [25–27]. PROG also upregulates the expression of antiapoptotic mitochondrial proteins such as Bcl-2. PROG may affect the expression of these mitochondrial proteins through activation of the ERK signaling pathway, which phosphorylates the cAMP response element binding protein (CREB), upregulates Bcl-2, and is known to provide improved resistance to ischemia [5]. PROG and its metabolites have also been demonstrated to modulate MAPK and PI3K signaling in the hippocampus, hypothalamus and cerebellum of ovariectomized rats in vivo [28,29]. Furthermore, PROG has been demonstrated to reverse alterations in mitochondrial respiration [30] and to normalize the expression of Na’,K’-ATPase in experimental autoimmune...
encephalomyelitis and models of nerve and spinal cord crush injury [31–33]. Since both respiration and Na⁺,K⁺-ATPase are important components in the cascade that leads to energy failure and loss of ionic gradients, we believe that this regulation of cellular metabolism is a key step in the prevention of cell death and the attenuation of secondary injury.

PROG has also been demonstrated to reduce inflammation, a significant mechanism of secondary injury, and is known to reduce microglial activation, as well as production of proinflammatory cytokines such as TNF-α and IL-1 [9,34]. This reduced inflammatory response is not only beneficial in the injury penumbra but has significant systemic effects, since TBI-associated systemic inflammation can often lead to multiorgan failure and infection [13] (mechanisms that are, in addition to cerebral edema, a frequent proximal cause of death after brain injury). Similar findings have been reported by Pan and colleagues, who found that following TBI in rats, expression of NF-κB, p65 and TNF-α were markedly decreased if the animals were administered PROG injections soon after the injury [35]. Measurement of brain edema, lesion volume and behavioral outcomes also revealed that PROG was effective in reducing the severity of the damage. Furthermore, in a series of studies, Chen et al. demonstrated that treatment with PROG reduces the expression of inflammatory cytokines, not just in the damaged brain, but also systemically in non-neuronal tissue such as the gut, spleen and intestine [17,36–38]. These findings support the systemic nature of brain injury and the fact that PROG acts at multiple receptor sites to control inflammation and improve functional outcomes (see also [39,40]).

Furthermore, there is evidence that PROG treatment after TBI reduces lipid peroxidation. The mechanisms of this action are not completely understood [6], but it is thought to occur in the upregulation of antioxidant enzymes such as superoxide dismutase [41]. Reducing the damage caused by reactive oxygen species and nitrogen species is known to improve cell survival in the penumbra of the injury by maintaining membrane integrity, and also helps to maintain the BBB by limiting oxidative damage to the capillary endothelium. PROG also helps maintain BBB function by upregulating P-glycoprotein, an efflux pump transporter and marker of BBB health that serves to eliminate xenobiotic and toxic substances, which consist of inflammatory cytokines and reactive oxygen species–producing compounds in the case of traumatic injury [42].

These molecular mechanisms – decreased inflammation and lipid peroxidation, maintenance of BBB integrity and improved ionic stability – all help to reduce cerebral edema after TBI [6]. Recent findings also indicate that PROG regulates the expression of aquaporin-4, a water channel expressed in astrocyte endfeet that is thought to be important for the development of edema [43]. Since brain swelling is one of the final neurological causes of mortality after TBI, this is an important concern for the clinical management of patients with brain injury.

**Summary of progesterone effects on receptors**

Although many of the molecular effects of PROG in neuroprotection have been described, it is still not known whether the classical intranuclear receptors are responsible for all of the hormone’s effects. In this review and others, perhaps contrary to the opinions of colleagues convinced that PROG can act only at a single receptor site, we have stressed the hypothesis that PROG has multiple beneficial effects that cannot be attributed solely to the PROG intranuclear receptor. For example, VanLandingham et al. have demonstrated that the enantiomer of PROG can substantially reduce cerebral edema following TBI without being able to bind to PROG receptor [44]. PROG and its metabolites, such as allopregnanolone, can reduce excitotoxicity at the NMDA receptor [45] and possibly decrease the incidence and severity of post-traumatic epilepsy by acting to potentiate γ-aminobutyric acid type A (GABAₐ) receptors to release more inhibitory neurotransmitters, thus acting, in some respects, similar to barbiturates and other anesthetics (e.g., see [49]). Studies in PROG receptor-knockout mice have demonstrated that the hormone can still exert anxiolytic and anesthetic effects, even in the absence of the PROG receptor. This probably occurs through the hormone’s metabolism to allopregnanolone and its actions on the GABAₐ and σ-1 receptors [46–48].

Covey and associates have published excellent and detailed reviews describing how neurosteroids interact with the GABAₐ receptor sites [49] (see [50] for more detail). With regard to mediating inflammatory reactions, PROG utilizes the glucocorticoid and PROG receptors to inhibit the production of nitrite and cytokines, such as IL-12, whereas synthetic progestins do not have this effect [51]. After brain injury, it also appears that PROG can modulate the activation of Toll-like receptors, which in turn affect the expression of inflammatory signaling factors, such as...
In neuroprotection, intracellular calcium regulation and homeostasis are important for cell survival after injury. Hwang et al. found that PROG influences the activity of inositol 1,4,5-trisphosphate receptors, which in turn regulate the levels of intracellular calcium in cultures of primary hippocampal neurons [52]. Some researchers argue that PROG can have both inhibitory and excitatory actions depending on whether the hormone is acting in the peripheral nervous system or CNS. For instance, Viero and Dayanithi demonstrated that PROG could produce substantial calcium influx by activating GABA and oxytocin receptors in the hypothalamus and supraoptic nucleus in neonatal rodents, but they observed the opposite effects in embryonic dorsal root ganglion, where GABA inhibited calcium influx [53]. The very rapid effects of PROG (such as its effects on inflammation and edema) are likely to be non-genomic and triggered at the cell surface. Recent experiments have identified a number of membrane PROG receptors, such as 25-Dx, or membrane PROG receptor-α, which when activated can stimulate the formation of new synapses and dendrites—important components of repair after CNS damage [54]. Guennoun et al. demonstrated that 25-Dx expression is substantially increased in neurons and astrocytes after both spinal cord and brain injuries, whereas the classical intranuclear receptor was actually downregulated [55]. It is also interesting to note that the 25-Dx receptor is also very abundant in the hypothalamus, where growth hormone expression plays a role in CNS repair in response to cerebral injury.

From even this very cursory review of PROG receptor mechanisms, it is apparent that neurosteroid receptor mechanisms and their role in CNS repair are complex. It is well beyond the scope of this update to provide full details, but some excellent reviews are available [5,21,56–58].

**Progestrone in aging & brain injury**

Although TBI affects all age groups, it is an especially significant health problem for those aged over 70 years, where both the incidence of hospitalizations and mortality rates due to TBI are highest [59]. While deaths resulting from TBI have been reduced in most demographics with improvements in safety, they have increased significantly in the older population. The elderly are subject to physiological and metabolic alterations that can affect recovery after major trauma, and therefore need to be considered specifically with regard to both treatment modalities and characteristic underlying physiology.

**Something new to consider: traumatic brain injury, progesterone, aging & vitamin D deficiency**

In a recent study, our laboratory tested the effectiveness of PROG in attenuating the acute inflammatory response after TBI in aged rats [42]. We observed significantly reduced levels of inflammatory markers, such as TNF-α, IL-6, NF-κB-p65 and COX-2, at 24, 48 and 72 h after injury. In addition, PROG reduced cell death, as measured by caspase-3 levels, and improved BBB integrity, as measured by P-glycoprotein levels. These molecular data were correlated with reduced cerebral edema and improved functional activity, all of which was consistent with the beneficial effects of PROG after TBI recorded repeatedly in younger animals [47,25,26,30,35,36,37]. The optimal doses and duration of treatment were also similar to those observed in younger animals, suggesting that PROG and its metabolites may be effective as a treatment for TBI across the life cycle.

In addition to advanced age itself, other factors that may affect the severity of TBI in the elderly include systemic issues, such as cardiovascular disease and atherosclerosis, hypertension, diabetes, kidney disease, cancer and hyperparathyroidism [60]. All these conditions can individually affect the response to injury, and each has also been recently associated with insufficient serum levels of vitamin D [61,62], suggesting that vitamin D deficiency may pose a risk factor for TBI severity in the elderly. This is important since vitamin D deficiency has been noted in all segments of the population, but especially in the elderly [61] and hospitalized individuals [63,64].

In another recent study, we examined the effects of vitamin D deficiency on acute inflammation in aged rats after TBI [65]. Our results demonstrated that, in both injured and uninjured aged rats, vitamin D deficiency can significantly elevate the levels of inflammatory markers (TNF-α, IL-1β, IL-6 and NF-κB-p65), increase cell death and affect short-term behavior. This acute effect is important in translating these results to the human population since acute inflammation has been strongly linked to survival in human trauma patients [66]. Surprisingly, vitamin D deficiency also attenuated the beneficial effects of PROG administration after TBI, although it was possible to overcome this through coadministration with 1,25-dihydroxyvitamin D$_3$ (VDH), the active form of vitamin D (Figure 1). Considering the prevalence of vitamin D deficiency in the elderly human population, these results could have significant consequences for clinical practice. They
also suggest that treatments with multiple drugs that affect different mechanisms of CNS injury and repair may be effective in the management of TBI and other CNS disorders.

**Progesterone & combination therapies**

The past two decades have seen the failure of many Phase II and III clinical trials for moderate and severe TBI, despite the fact that over 130 different compounds had initially shown promise in preclinical models of injury [67]. One of the major reasons suggested for this disappointing state of affairs is the fact that the complex and varied mechanisms observed in TBI cannot be adequately addressed by single-drug therapies targeted to a specific mechanism or receptor site. In contrast to the clear outcomes and well-circumscribed injuries of experimental animal models of injury, clinicians are well aware that human TBI pathophysiology is highly variable and heterogeneous, and affects many organs and tissue systems outside the brain itself. There is growing recognition that pharmacotherapies targeting more than one mechanism, or the same mechanisms differently, or at different time-points, may be more effective than the commonly applied ‘monotherapies’ [67]. This idea is already prevalent in the treatment of diseases such as HIV/AIDS and tuberculosis, where multitherapeutic approaches that target different parts of the disease process, act at different sites, and synergistically increase activity have become the standard. A similar approach has been suggested for TBI owing to its complex manifestation in human patients [67]. A number of treatments other than PROG have been suggested as the ‘main’ component of a potential combination therapy, including citicoline, erythropoietin, hypothermia, cyclosporin A, statins and hypertonic saline [67], all of which have shown some promise in preclinical experiments. A literature review and our previous data with vitamin D deficiency suggest that VDH may also be a good candidate for combination therapy [68]. VDH is a neuroactive steroid known to be neuroprotective, with functional attributes similar to PROG, although it also has a number of divergent actions. It is also cheap, easy to administer and readily available. Based on our laboratory data, we think that a combination of PROG and VDH could lead to improved neuronal and cellular repair and recovery after injury, perhaps with less dosing and duration of treatment than with either agent administered alone.

A recent paper from our laboratory supports this approach [69]. We found that in cultured cortical neurons challenged with glutamate, both VDH and PROG individually demonstrated neuroprotection, but combined treatment was more effective than treatment with either compound alone. Significantly, the most effective combination dosages were different from the individual

**Figure 1. Effects of vitamin D deficiency on short-term behavior after traumatic brain injury.** Locomotor behavior associated with acute inflammation in aged rats 72 h after TBI. The dark bars represent vitamin D-sufficient animals and the light bars represent vitamin D-deficient animals. It should be noted that only a combination of PROG (16 mg/kg) and VDH (5 μg/kg; D+PROG) returns behavior to sham-injured levels in deficient animals, whereas PROG alone is sufficient in nutritionally normal animals. This suggests that vitamin D deficiency exacerbates the injury and interferes with PROG treatment, but also that this effect can be reversed with acute VDH administration.

* p < 0.05 versus sufficient VH; ‡ p < 0.05 versus deficient VH.


Adapted with permission from [65].
‘best’ doses, suggesting synergy between the two drugs that would not be predicted based on treatment with either agent alone (Figure 2).

One reason for treating injury pathways with multiple compounds is the possibility that a single repair mechanism may be modulated through different signaling mechanisms. Another reason is that a single high-dose drug treatment could saturate receptors and result in loss of functional benefit, which can happen in the case of PROG, for example [70]. This problem might be overcome by activating a different pathway leading to neuroprotection [71]. As an example, PROG and VDH could each increase the activity of γ-glutamyl transpeptidase by different mechanisms (PROG receptor–PROG activity and vitamin D receptor–VDH activity), resulting in higher overall antioxidant capacity. The possibility of amplifying a neuroprotective effect by combinatorial therapy is worth further exploration.

In summary, there is now substantial preclinical and clinical evidence that PROG can have salutary effects on morphological and functional recovery after TBI. Over the last 5–6 years and even since the first review of PROG in Future Neurology, much has been discovered about its specific mechanisms of repair. A comprehensive discussion of the many receptor mechanisms involved in neurosteroid and vitamin D actions is beyond the scope of this update, but there are a number of recent articles on this subject [71–76].

It is unfortunate that, thus far, most clinical trials for TBI treatments have failed, leading to considerable pessimism that a successful treatment will ever be found. Nonetheless, the fact that the NIH is supporting a Phase III multicenter trial using PROG for moderate-to-severe TBI is encouraging [103]. Unlike all other agents recently tested, PROG is a naturally occurring hormone with a high safety profile as demonstrated in two Phase II trials, both of which have shown substantial benefit in reducing mortality and improving functional outcomes after TBI [2,3].

Future perspective

As the Phase III trial goes forward, investigators and clinicians are asking: what’s next? One of the key clinical areas now under investigation in our laboratory is ischemic stroke. There is a small amount of literature demonstrating that in animal models of ischemic injury, PROG and its metabolite allopregnanolone can reduce the size of the ischemic infarct and produce functional benefits [6]. Less is known about PROG in the treatment of military blast injuries to the head and in pediatric traumatic or hypoxic brain injury, although these are also important potential applications. There is also growing interest in determining whether neurosteroids could be applied to more chronic neural disorders, such as

Figure 2. Effects of combination progesterone and 1,25-dihydroxyvitamin D treatment on cell survival in vitro. The effects of progesterone and VDH combination treatment on glutamate-induced MTT reduction in rat primary cortical neurons. Cells were pretreated with different combinations of progesterone (20 µM) and VDH (doses shown as ‘D’ nM) for 24 h and subsequently exposed to glutamate (0.5 µM) for 24 h. Note the significant synergistic effect between P and D20, which is not observed with all combination dosages. *p < 0.001 versus VH; †p < 0.001 versus CTRL; ‡p < 0.01 versus P20 alone.

CTRL: Control; P: Progesterone; VDH: 1,25-dihydroxyvitamin D; VH: Vehicle.

Adapted with permission from [69].
amytrophic lateral sclerosis, multiple sclerosis or Parkinson’s disease. This is certainly a topic for further review and discussion, but far too preliminary at this stage to consider for clinical trial.

A key point to keep in mind is that this hormone has characteristics that are protective of the fetus during gestation and of the nervous system when administered exogenously throughout the spectrum of development, including old age. It can be argued that PROG works as a neuroprotective agent because, to a great extent, many of the processes involved in brain repair are similar to growth and organizational processes occurring in early life. This notion is admittedly debatable, but it is a hypothesis that can be subjected to experimental testing and verification. If only because not much else in the field of brain injury has been shown to work as well, the hormone and its related metabolites deserve continued experimental attention and clinical evaluation.

**Executive summary**

**Background**
- Recent data demonstrates that neurosteroids, specifically progesterone (PROG) and some of its metabolites, are neuroprotective in experimental models of traumatic brain injury (TBI).
- Two recent Phase II clinical trials of PROG as a treatment for TBI have also shown promise. A multicenter Phase III trial is now underway to determine clinical effectiveness.

**Mechanisms & systems**
- PROG has been demonstrated to protect neurons from ischemic injury and to decrease the size of a lesion or ischemic infarct. These actions are a result of the hormone’s effects on maintenance of mitochondrial functions, increased prosurvival signaling, reduced inflammatory reactions, apoptosis and reactive oxygen species, stimulation of myelin synthesis and restoration of the BBB. These effects are systemic and not limited to the CNS injury itself.

**Receptors**
- PROG acts at a number of different sites to enhance neuroprotection.
- PROG and its metabolites can upregulate gene activity through their direct actions on the PROG intranuclear receptor, but they can also act at the NMDA receptor to reduce excitotoxicity and at the GABA \( \alpha \) and \( \sigma \) receptors to release more inhibitory neurotransmitters and thus block post-traumatic seizure activity.
- PROG modulates Toll-like receptors, and this action affects the expression of inflammatory factors and cytokines such as NF-\( \kappa \)B and IL-1, among others.
- The hormone also acts on membrane receptors, such as 25-Dx, which alters calcium toxicity.

**Progesterone, aging & vitamin D**
- Vitamin D deficiency, especially in older subjects, leads to increased expression of inflammatory factors and reduces the benefits of PROG treatment for TBI. Combining vitamin D with PROG leads to better functional outcomes than either treatment alone.
- Both PROG and vitamin D exert their effects through a variety of metabolic pathways and receptor mechanisms activated in the secondary injury cascade after TBI. Both can promote neural survival, repair and better functional outcomes.

**Progesterone & combination therapies**
- Many clinical trials have failed because they do not address the complexity of TBI as a systemic disease and the varied mechanisms involved in tissue damage and repair. There is growing recognition that combination therapies, such as vitamin D and PROG, can have more beneficial effects in combination than when administered alone. This is a relatively new area of research that requires more attention.

**Future perspective**
- The effects of neurosteroids on TBI may also prove beneficial in several other applications. In all cases, much work remains to be done:
  - Ischemic stroke: a few recent studies in animal models suggest that PROG and allospregnanolone can reduce infarct size and produce functional benefits;
  - Pediatric traumatic or hypoxic brain injury: some animal models are now being studied.
- PROG’s effects on remyelination and inflammation may make it a therapeutic candidate for neurodegenerative and other chronic CNS disorders, such as amyotrophic lateral sclerosis, multiple sclerosis and Parkinson’s disease. This is a very new area where work has only just begun.

**Financial & competing interests disclosure**

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* of interest
** of considerable interest


**Along with [3]**, presents results of Phase II trials using progesterone to treat moderate-to-severe traumatic brain injury (TBI).


**Along with [2]**, presents results of Phase II trials using progesterone to treat moderate-to-severe TBI.


**Excellent overview of progesterone receptor mechanisms in the CNS.**


* Provides a detailed review of the potential uses of progesterone in the treatment of a spectrum of CNS disorders.


* Recent comprehensive review comparing the uses of progesterone and estrogen in the treatment of TBI and stroke.


* Written for physicians wishing to learn more about progesterone in the treatment of TBI. This review includes a very brief overview of mechanisms of action.


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Umbilical cord blood cell and granulocyte-colony stimulating factor: combination therapy for traumatic brain injury

Keywords: combination therapy • granulocyte-colony stimulating factor • human umbilical cord blood • traumatic brain injury

Traumatic brain injury (TBI) is a major public health problem associated with death and permanent disability worldwide [1]. Both acute and chronic symptoms accompany TBI, with survivors suffering from progressive post-TBI pathological manifestations such as neuroinflammation coupled with behavioral dysfunctions including sensory motor deficits, learning and memory impairments and a range of neuropsychiatric symptoms including anxiety, depression and aggression [2,3]. At present, there is significant unmet need for clinically efficacious therapies for TBI [4].

Stem cell transplantation has been shown to be an effective regenerative therapy for functional and physiological improvement in animal models of brain disorders [5]. Among various types of stem cells, adult stem cells are of interest as they circumvent ethical and moral problems, as well as teratogenic and oncogenic risks usually associated with transplantation of embryonal- or fetal-derived stem cells [6]. In particular, a number of groups have focused on the potential of human umbilical cord blood (hUCB)-derived stem cells as a graft source for various intractable neurological disorders (e.g., stroke, Parkinson’s disease and Huntington’s disease, among others; for review see [6]). Moreover, clinical trials have been performed to determine the efficacy of hUCB stem cells in cerebral palsy, inborn metabolic disorders and stroke (for review see [7]).

hUCB stem cells have also been used experimentally in animal models of TBI. In some studies, transplanted cells derived from the mononuclear fraction of hUCB have been shown to confer neuroprotection by decreasing inflammation and brain tissue loss, promoting neurogenesis and rescue of neurological dysfunctions [8,9]. However, an issue associated with hUCB transplantation, as with utilizing other cell types, involves limited regenerative capacity of transplanted stem cells due to the inhospitable microenvironment of the injured brain [10]. Therefore, a combination treatment that creates a conducive host–tissue microenvironment for the transplanted stem cells may improve the therapeutic outcome.

The granulocyte-colony stimulating factor (G-CSF), an essential member of the hematopoietic growth factor family, has been shown to be neuroprotective in animal models of stroke [11] and it also improved cognition, reduced central and peripheral inflammation and enhanced neurogenesis in models of Alzheimer’s disease [12]. The safety and efficacy of G-CSF for acute ischemic stroke has already been explored in clinical studies [13,14]. However, G-CSF treatment in animal models of TBI produced discrepant results. While some studies reported improvement of TBI-associated behavioral and histological impairments, others found minimal effects of G-CSF on functional and neurological outcomes in animals subjected to TBI [15,16].

In a recent study, Acosta et al. [17] tested the putative benefits offered by combination therapy in TBI by investigating whether there is enhanced behavioral and histological improvement after transplantation of hUCB and co-administration of G-CSF in a controlled cortical impact model of moderate...
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TBI in adult rats. Their study revealed that while monotherapy with hUCB or G-CSF promoted behavioral recovery, combined therapy of hUCB plus G-CSF enhanced further functional improvement exerted by the latter treatment regimen. Moreover, the effects of combination therapy were longer lasting than those exerted by hUCB transplantation or G-CSF administration alone. Complementary brain-repair processes distinctly or mutually solicited by these two therapies could have mediated improved functional recovery exerted by combination treatment of hUCB plus G-CSF. Endogenous stem cells mobilized by G-CSF from the bone marrow to the peripheral blood (and ultimately to the site of injury) [11–14], growth factors secreted by hUCB grafts and the potential graft–host integration that leads to reconstruction of synaptic circuitry [18] altogether could have facilitated the regenerative mechanisms exerted by the combination therapy, but not by monotherapy. Further studies are needed to clarify this multipronged mechanism of action by the combination hUCB plus G-CSF therapy.

Acosta et al. also performed immunohistochemical staining with OX-6, which labels MHCII+ cells (putatively activated microglia), to determine the extent to which combination therapy of hUCB plus G-CSF ameliorated TBI-induced neuroinflammation in gray and white matter areas [17]. In parallel to the improved behavioral recovery with combination therapy, the investigators found that combined hUCB plus G-CSF treatment resulted in more profound reduction of TBI-induced upregulation of MHCII+ cells in the cortex, striatum, thalamus, subventricular zone and the dentate gyrus of the hippocampus, compared with hUCB and/or G-CSF monotherapy. Moreover, whereas hUCB transplantation or G-CSF treatment alone suppressed activated MHCII+ cells in the corpus callosum and fornix, combined therapy of hUCB plus G-CSF decreased the number of MHCII+ cells not only in the corpus callosum and fornix, but also in the cerebral peduncle [17].

“...combined therapy of human umbilical cord blood plus granulocyte-colony stimulating factor synergistically dampened traumatic brain injury-induced neuroinflammation while significantly enhancing endogenous neurogenesis and reducing hippocampal cell loss.”

That combination therapy enhanced the therapeutic outcome in TBI begs the question whether synergistic effects were produced by hUCB plus G-CSF in attenuating TBI-induced impairment in endogenous neurogenesis and hippocampal cell loss. In animal models of TBI, as well as in preclinical stroke and aging studies, hUCB treatment has been shown to decrease inflammation and facilitate neurogenesis and angiogenesis [19,20]. In a similar fashion, treatment with G-CSF has been shown to modulate neurogenesis in TBI, as well as in other neurodegenerative disorders (e.g., hypoxic injury and Alzheimer’s disease, among others) [12]. The findings from the studies of Acosta et al. showed that reduction in neuroinflammation exerted by hUCB plus G-CSF therapy coincided with elevated neurogenesis in dentate gyrus and subventricular zone while increasing the survival of CA3 neurons in TBI rats. Altogether, the results from the above-mentioned studies indicate that combined therapy of hUCB plus G-CSF synergistically dampened TBI-induced neuroinflammation while significantly enhancing endogenous neurogenesis and reducing hippocampal cell loss [17].

“A complementary interaction between hUCB and G-CSF may also explain the widespread effects of this combination therapy in diverse brain regions. As mentioned above, G-CSF displays the capacity to mobilize stem cells from the bone marrow to the peripheral blood, and the mobilized cells have been shown to infiltrate injured tissues promoting self-repair of neurons [11–14]. Additionally, G-CSF permeates the blood–brain barrier and acts upon neurons and glial cells through the G-CSF receptor [21]. Among the many beneficial effects of G-CSF receptor activation in neurons and glia include downregulation of proinflammatory cytokines and increased neurogenesis [22,23]. Moreover, the combination of hUCB plus G-CSF may promote stemness maintenance, and under appropriate conditions, guide neural lineage commitment of hUCB [24]. On the other hand, mobilized bone marrow cells and hUCB cells may also exert their therapeutic benefits via a paracrine mechanism, in other words, transplanted cells secrete trophic factors, growth factors, chemokines and immune-modulating cytokines to the injured milieu, in line with the concept of ‘bystander effects’ of transplanted stem cells [25,26]. In summary, a receptor-mediated transport mechanism coupled with paracrine effects of transplanted cells may underlie extensive influence of combination therapy of human umbilical cord blood plus granulocyte-colony stimulating factor in diverse brain regions...”
and increased cell survival in TBI rats that received hUCB plus G-CSF.

**Conclusion & future perspective**

In the clinic, chronic TBI has been recently recognized as a progressive cell death process rather than an acute event, characterized by a worsening histopathology with limited therapeutic opportunities [27]. The study of Acosta et al. demonstrated long-lasting recovery of motor functions and more robust attenuation of neuroinflammation accompanied by enhanced neurogenesis and profound rescue against neuronal cell death in TBI rats given hUCB plus G-CSF. This study showed how stand-alone therapies (i.e., hUCB and G-SCF) could overcome their therapeutic limitations in chronic TBI when synergy is accomplished through combination therapy. That TBI-associated cell death cascades require multipronged regenerative processes highlights the need for combination therapy targeting different cell death or survival pathways. Such multiple biologic therapeutic approaches open new research directions and clinical applications for TBI. Considering that hUCB and G-CSF present with solid safety and efficacy profiles as stand-alone therapies, their combination therapy appears indicated for limited clinical trials in TBI.

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