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Neuroprotective effects of progesterone in traumatic brain injury: blunted in vivo neutrophil activation at the blood-brain barrier

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Abstract

BACKGROUND—Progesterone (PRO) may confer a survival advantage in traumatic brain injury (TBI) by reducing cerebral edema. We hypothesized that PRO reduces edema by blocking polymorphonuclear (PMN) interactions with endothelium (EC) in the blood-brain barrier (BBB).

METHODS—CD1 mice received repeated PRO (16 mg/kg intraperitoneally) or vehicle (cyclodextrin) for 36 hours after TBI. Sham animals underwent craniotomy without TBI. The modified Neurological Severity Score graded neurologic recovery. A second craniotomy allowed in vivo observation of pial EC/PMN interactions and vascular macromolecule leakage. Wet/dry ratios assessed cerebral edema.

RESULTS—Compared with the vehicle, PRO reduced subjective cerebral swelling ($2.9 \pm .1$ vs $1.2 \pm .1$, $P < .001$), PMN rolling (95 ± 1.8 vs 57 ± 2.0 cells/100 $\mu\text{m}/\text{min}$, $P < .001$), total EC/PMN adhesion ($2.0 \pm .4$ vs $.8 \pm .1$ PMN/100 μm , $P < .01$), and vascular permeability ($51.8\% \pm 4.9\%$ vs $27.1\% \pm 4.6\%$, $P < .01$). TBI groups had similar a Neurological Severity Score and cerebral wet/dry ratios ($P > .05$).

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CONCLUSIONS—PRO reduces live pericontusional EC/PMN and BBB macromolecular leakage after TBI. Direct PRO effects on the microcirculation warrant further investigation.

Keywords

Intravital microscopy; Neutrophil; Endothelium; Blood-brain barrier; Traumatic brain injury; Progesterone

Traumatic brain injury (TBI) is the leading cause of death and disability in young Americans, with 1.7 million cases occurring yearly.¹ In severe TBI, intracranial hypertension occurs when the brain swells against the noncompliant dura and, if uncontrolled, may cause brain herniation and ultimately death. Brain edema after TBI may occur through a leaky blood-brain barrier (BBB) resulting from the initial impact but also from ongoing cerebrovascular inflammation and injury.² Although several neuroprotective therapies have shown promise in animal studies, none have been successful clinically, and to date no therapy exists to curb the ongoing progression of cerebral injury after the initial trauma.

In the last decade, increasing evidence has emerged, suggesting that polymorphonuclear neutrophil (PMN) and endothelial cell (EC) activation in the microcirculation may sustain the persistent microvascular disruption that evolves in the hours after injury.^{1,2} In systemic circulation, PMNs pass from the vasculature to tissue in a series of steps involving surface adhesion receptors on both the PMN and EC and that result in the PMN reaching the site of injury to perform cytotoxic and phagocytic functions.³ In some cases, neutrophils may become inappropriately activated and inappropriately release cytotoxic substances intravascularly, resulting in ongoing vascular and organ injury.⁴ Decreasing this inflammatory host response at the BBB may potentially decrease secondary brain injury and improve clinical outcomes.

Progesterone (PRO), a potent sex and neurosteroid, may improve survival and cognitive recovery after TBI and is currently being investigated in a phase 3 clinical trial.^{5,6} Known effects of PRO in TBI include neuronal apoptosis and blunting of oxygen-free radical and inflammatory cytokine production.⁵ It remains unknown whether PRO affects circulating PMNs and ECs in the BBB and how this relates to microvascular inflammation and injury. We hypothesized that PRO visibly reduces EC/PMN interaction in the BBB microcirculation, decreases cerebral edema, and results in greater functional recovery after TBI in a murine model.

Materials and Methods

Animal model: craniotomy and traumatic brain injury

All experiments were performed after approval by the Institutional Animal Care Committee of the University of Pennsylvania. Male CD1 mice (25 to 30 g) were housed in standard facilities for 5 to 7 days before study. On day 1, mice were anesthetized with intraperitoneal ketamine, xylazine, and acepromazine (100, 10, and 1 mg/kg, respectively) followed by .05% isoflurane via a nasal cone. Animals were placed prone on a warming pad covering a stereotactic frame (Stoelting, Wood Dale, IL), and after scalp incision, a left-sided, 4-mm

craniotomy was created with a trephine centered between the bregma and lambda (Fig. 1). The left parietotemporal cortex was then injured by controlled cortical impact (CCI) (AMS201; AmScien Instruments, Richmond, VA),⁷ which resulted in an injury consistent with severe TBI (3-mm-diameter impactor tip, impact velocity of 6 m/s, and cortical deformation of 1.0 mm). Animals were awakened and returned to their cages.

Experimental protocol and study groups

Thirty animals were randomized into the following 3 groups: CCI + PRO (n = 10), CCI + vehicle (VEH) (n = 10), and sham (craniotomy, no CCI; n = 10). Both PRO (16 mg/kg in 22.5% cyclodextrin) and VEH (22.5% cyclodextrin) (Sigma Aldrich, St Louis, MO) were given intraperitoneally 30 minutes after CCI and every 12 hours thereafter for 36 hours. Animals were then prepared for intravital microscopy.

Animal activity and neurologic recovery

Every 12 hours postoperatively rodent neurologic recovery was graded using the modified Neurological Severity Score,⁸ a composite of motor, sensory, reflex, and balance tests graded in a scale from 1 to 18.

In vivo assessment of gross cerebral swelling and microcirculation

Thirty-six hours after craniotomy, animals received ketamine, xylazine, and acepromazine intraperitoneally and underwent the placement of a left external jugular vein line (PE-10; BD Biosciences, Sparks, MD) for the administration of rhodamine and fluorescein isothiocyanate (FITC)-labeled albumin. Mice were again placed on the stereotactic frame, and their previous scalp incision was reopened to allow visualization of the exposed cerebral cortex. Gross cerebral swelling out of the craniotomy was scored subjectively by 1 of the authors (MAM) by determining the degree of bulging of the cerebral cortex into the skin incision (0 = no bulging, 3 = severe bulging). A second 2.5-mm craniotomy was then created anterior to the first craniotomy for in vivo video microscopy (Fig. 1A). A 5-mm cover slip (Fisher Scientific, Pittsburgh, PA) was secured over the second craniotomy with ethyl cyanoacrylate glue between cover slip and bone. After transferring to an in vivo video microscope (ECLIPSE FN1; Nikon Instruments, Melville, NY), animals received a 50- μ L intravenous bolus of .3% rhodamine 6G (300 μ g/mL in .9% saline .05-mL bolus) to fluorescently label circulating PMNs. Footage of the pial microcirculation was recorded under a 590-nm epiillumination emission filter using a digital camera (Quan-tEM; Photometrics, Tucson, AZ) and saved to a hard drive for subsequent analysis by a blinded observer. Two to 3 non-branching postcapillary venules (25 to 50 μ m in diameter and 100 μ m in length) were selected for 1-minute recordings in each animal. After 30 minutes, 50 mg/kg FITC-labeled albumin (Sigma Aldrich) was administered intravenously and, using the same pial regions, venular albumin leakage was visualized through a 488-nm fluorescent filter.

Quantification of polymorphonuclear neutrophil/endothelial cell interactions and microvascular permeability

Video recordings were imported into digital analysis software (NIS-Elements, Nikon Instruments) for offline counting of 4 types of EC/PMN interactions by a blinded observer: (1) PMN rolling: the mean number of PMN crossing a 100- μ m venular segment; (2) PMN preadhesion: immobile PMN at the initiation of the 30-second recording period; (3) PMN adhesion, stationary PMN for at least 30 seconds during the recording period; and (4) total PMN adhesion: sum of (2) and (3). Static images from FITC-labeled albumin recordings also were analyzed offline, and fluorescence was measured in 3 regions within the vessel (venular intensity) and outside the vessel wall (perivenular intensity) (Fig. 2B). The ratio of venular intensity to perivenular intensity was averaged to determine the permeability index for the given vessel.

Wet to dry assessment of tissue edema

After the animals were killed, animal brains were excised, hemispheres were split, and each was weighed immediately (wet weight) and after 72 hours of drying at 70 °C (dry weight). Brain edema was determined by wet-to-dry ratios $([\text{wet weight} - \text{dry weight}]/\text{wet weight})$ in ipsilateral and contralateral cerebral hemispheres.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean. Differences between groups were compared using analysis of variance with Bonferroni correction. A P value $<.05$ was considered significant.

Results

Animal recovery and gross brain swelling at 36 hours

Both TBI groups lost significant body weight after 36 hours compared with the sham group (mean = 6.3%, $P <.01$). The overall mean neurologic function scores (modified Neurological Severity Score) were similar between treatment groups (PRO: $16.0 \pm .2$ vs VEH: $15.9 \pm .3$, $P = .67$) and significantly worse than in sham animals ($18 \pm .0$, $P <.001$ vs either PRO or VEH). Gross cerebral swelling through the CCI craniotomy was significantly greater in VEH than PRO animals ($2.9 \pm .1$ vs $1.2 \pm .1$, $P <.001$), but both had more swelling than sham animals ($.0 \pm .0$, $P <.0001$, sham vs either PRO or VEH).

Blood-brain barrier microcirculation: polymorphonuclear neutrophil rolling and adhesion to endothelium

Thirty-six hours after CCI, PMN rolling in the pericontusional microvessels was greater in the VEH group (95 ± 1.8 cells/100 μ m/min) than in the PRO (57 ± 2.0 cells/100 μ m/min, $P <.001$) and sham groups (48 ± 4.0 cells/100 μ m/min, $P <.001$) (Fig. 3). PMN rolling was greater in PRO than sham animals, but this did not reach significance ($P = .07$). New PMN adhesion was similar between groups (mean $.3 \pm .1$ cells/100 μ m, $P = \text{not significant}$) (Fig. 4). Adherent PMN upon the initiation of counting (preadhesion) in PRO animals ($.5 \pm .1$ cells/100 μ m) were similar to sham animals ($.75 \pm .2$ cells/100 μ m, $P = 1.0$). Preadhesion in

VEH ($1.6 \pm .3$ cells/100 μm) was greater than in PRO animals ($P = .005$) but not sham animals ($P = .06$). Total PMN adhesion to EC was significantly higher in TBI treated with VEH ($2.0 \pm .4$ cells/100 μm) than with PRO ($.8 \pm .1$ PMN/100 μm , $P = .01$) or sham ($.9 \pm .2$ PMN/100 μm , $P < .05$). Total adhesion was similar in the sham ($.9 \pm .2$ cells/100 μm) and PRO groups ($P = 1.0$).

Microvascular permeability

Ten minutes after intravenous injection of a macromolecule (FITC albumin), live vascular leakage was significantly higher in VEH ($51.8\% \pm 4.9\%$) than PRO ($27.1 \pm 4.6\%$, $P < .01$), which was similar to that in sham ($22.0\% \pm 4.3\%$, $P = .4$) (Fig. 2).

Brain edema

Although wet to dry ratios in both VEH (33.0 ± 4.5) and PRO (24.9 ± 3.9) groups showed less ipsilateral hemisphere edema than in sham animals (48.2 ± 2.2 , $P = .01$ and $P < .001$, respectively), there were no differences between the 2 treatment groups ($P = .2$). In the contralateral hemisphere, wet to dry ratios in PRO (32.6 ± 3.0) but not VEH animals (41.1 ± 4.2) showed less edema than sham animals (45.4 ± 2.1 , $P < .01$, $P = .13$, respectively).

Comments

PRO is currently being investigated in a large multicenter randomized controlled trial evaluating moderate and severe TBI (Progesterone for Traumatic Brain Injury: Experimental Clinical Treatment: Phase III Clinical Trial [ProTECT III], clinical trials.gov NCT00822900). This trial follows 2 antecedent prospective human trials showing PRO to reduce mortality (severe TBI) and improve neurologic recovery (moderate TBI) at 3 and 6 months in survivors.^{5,6}

In the current study, in vivo observation of the rodent BBB 36 hours after TBI treated with PRO concurrently reduced live PMN/EC interactions and gross macromolecule leakage to sham levels. Grossly, PRO also reduced brain swelling.

The mechanisms by which PRO may be protective in TBI remain unclear and likely involve multiple cellular pathways. PRO is a potent neurosteroid synthesized in the central nervous system, and after exogenous intravenous administration, rapidly enters the brain, reaching equilibrium within 60 minutes. Using intraperitoneal administration of PROs in this and most other animal studies may not result in such a rapid intracerebral presence of the hormone. Several animal studies have shown that the administration of PRO after experimental TBI reduces neuronal apoptosis and limits gliosis.^{9,10}

Although the primary brain injury occurs at the time of cerebral impact, the subsequent ongoing cerebral injury that persists in the hours and days that follow constitutes secondary brain injury and is potentially modifiable by different management strategies. In the last decade, microvascular neuroinflammation implicating the PMN has emerged as an important contributor to secondary brain injury.¹¹ After trauma, an overzealous activation of PMNs may occur resulting in a self-perpetuating host response.¹² Within 12 hours, PMNs

are recruited to the disrupted cortical BBB.¹³ In vivo data suggest that BBB PMN recruitment is delayed⁷ and may not occur immediately after cortical impact.¹⁴

PMNs then follow a series of steps to migrate out of flow, first by L-selectin mediated rolling followed by PMN adhesion to EC through surface CD11b binding to EC Intercellular Adhesion Molecule - 1 (ICAM-1).³ Adherent PMNs then transmigrate through the vessel wall to perform physiologic cytotoxic and phagocytic functions. In acute injury, dysregulated activation may occur, resulting in unrelenting EC/PMN interactions and the indiscriminant PMN release of cytotoxic enzymes and O₂-free radicals that result in ongoing microvascular inflammation and injury rendering more permeable or “leaky.”¹²

Although we have previously found significant PMN recruitment to the BBB 36 hours after TBI,⁷ the current study suggests that PRO may significantly attenuate PMN rolling and adhesion to the endothelium. PRO may influence EC/PMN interactions in the BBB circulation in a variety of ways. Few studies have previously investigated the effects of PRO on the neutrophil. In 1 study, human PMNs exposed to PRO showed enhanced migration although this was in vitro and not in the context of circulating flow in a vessel.¹⁵ In bovine veterinary experiments, higher circulating PRO levels were correlated with reduced PMN respiratory (oxidative) burst and release of oxygen radicals.^{16,17} In particular, PRO reduces lipid peroxidation in neuronal cells in a dose-dependent fashion, while increasing mitochondrial glutathione levels, a critical oxygen radical scavenger.¹⁸ Lastly, PRO may blunt PMN adhesion receptor expression. In a clinical study, Orvieto et al¹⁹ found that women with higher PRO levels showed reduced PMN L-selectin expression. There are even less data about PRO effects on the endothelium. One study found blunted EC secretion of prostacyclin, which inhibits activation of circulating platelets and leukocytes.²⁰ In sum, these data suggest a direct effect of PRO, particularly on PMNs, that may have resulted in the observed reduction in EC/PMN interactions in the current study.

As seen in previous animal models of TBI, we found that PRO treatment reduced visible brain swelling^{21,22} and blunted the in vivo permeability of penumbral vessels.^{23,24} Although wet to dry measurements did not differ between groups, we found that PRO tended to reduce ratios of both ipsilateral and contralateral cerebral hemispheres compared with VEH. Interestingly, brains of sham animals had a greater wet weight than either injured group. This unexpected finding may relate to the CCI model, which creates a large devitalized hemisphere that potentially could not become edematous although no histologic analysis was conducted to confirm this.

Unlike other animal TBI studies,²⁵ we were unable to show improved neurologic recovery in animals receiving PRO. However, these investigators administered PRO for a duration that was 3 times longer (120 hours) and used the water maze test, which is a more sensitive assessment than the Neurological Severity Score.²⁶

This study has a number of important limitations. Sample sizes were small, and larger groups may have allowed trends in wet to dry differences to reach significance. Also, PRO or VEH was administered by the same operator of microscopic experiments. However, an independent person without knowledge of treatment performed offline PMN counting and

the determination of FITC-albumin leakage. An ideal complimentary evaluation would have been to analyze PMN respiratory burst and adhesion receptor expression in the different groups, but blood exposed to fluorophores yields imprecise flow cytometric measurements. Finally, the TBI model used gave animals an open head injury with the CCI craniectomy remaining decompressed during progression of the brain injury, and as such this may not be reflective of the more common closed head injury seen in blunt TBI patients.

This article adds important data to the existing knowledge of PRO effects in severe TBI. PRO attenuates BBB cellular interactions and the subsequent microvascular permeability that ensues. Further investigation in the direct effects of PRO on the endothelium and PMNs will be needed to confirm if this is a key mechanism of PRO benefits in severe TBI.

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Figure 1.

(A) Timeline of experiments. R6G, rhodamine. (B) Skull surface anatomy and location of controlled cortical CCI and microscopy craniotomies.

Figure 2.

(A) Percent leakage of FITC-labeled albumin 10 minutes after injection and 36 hours after TBI. The increased vascular permeability seen with VEH was not observed with PRO or in sham animals. * $P < .01$ vs sham. # $P < .01$ vs PRO. (B) Light intensity (grays) was measured 10 minutes after FITC-albumin administration in 3 distinct 70-μm regions within the vessel (Iv) and outside the vessel (Ip). Vessel permeability was calculated as mean Iv/mean Ip.

Figure 3.

(A) PMN rolling in the BBB was reduced to sham levels by PRO. * $P < .001$ vs sham. # $P < .001$ vs PRO. (B) PMN rolling and adhesion. White arrows indicate labeled PMN interacting with the endothelium.

Figure 4.

PMN adhesion to the endothelium in the BBB 36 hours after TBI. PRO reduced total adhesion to that found in uninjured sham animals primarily by reducing the number of adherent PMNs at the initiation of counting (preadhesion). * $P = .06$ vs sham. # $P = .01$ vs PRO. † $P < .05$ vs sham.