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Gender Differences in the Effect of Progesterone after Controlled Cortical Impact in Adolescent Mice: A Preliminary Study

Rebekah Mannix, MD, MPH^{1,2}, Jacqueline Berglass¹, Justin Berkner¹, Philippe Moleus¹, Jianhua Qiu, MD, PhD^{1,2}, Lauren Jantzie, PhD^{2,6}, William P. Meehan III, MD^{1,2,3,4}, Rachel M. Stanley, MD⁵, and Shenandoah Robinson, MD^{2,6}

¹Division of Emergency Medicine, Boston Children's Hospital, Boston Massachusetts

²Harvard Medical School, Boston Massachusetts

³Sports Concussion Clinic, Division of Sports Medicine, Boston Children's Hospital, Boston Massachusetts

⁴The Micheli Center for Sports Injury Prevention, Waltham Massachusetts

⁵Department of Emergency Medicine, University of Michigan Health System, Ann Arbor Michigan

⁶Department of Neurosurgery, Boston Children's Hospital, Boston Massachusetts

Abstract

Objective—While progesterone has been well studied in experimental models of adult traumatic brain injury (TBI), it has not been evaluated in pediatric models of TBI. Studying promising interventions in pediatric TBI is important because children have the highest public health burden of TBI. Therapies that are beneficial in adult TBI patients, however, may not be effective in the pediatric population.

Methods—Here, we evaluated whether progesterone administered after controlled cortical impact (CCI) improves functional and histopathological outcomes observed in 4 week old mice. We included male and female mice as the majority of prior studies have used male or reproductively senescent females.

Results—When compared to mice treated with vehicle alone, motor performance improved when injured male mice were treated with progesterone ($p < 0.001$), but worsened when female mice were treated ($p = 0.001$). Progesterone treatment had no effect on spatial memory performance or lesion volume in injured male or female mice.

Conclusions—These data suggest a gender specific effect of progesterone treatment after CCI in adolescent mice, and could inform clinical trials in children.

Keywords

traumatic brain injury; progesterone; adolescent; gender; neuroprotection

Address correspondence and reprint requests to Dr. Rebekah Mannix, Boston Children's Hospital, 300 Longwood Avenue, Boston MA 02115, USA; Rebekah.Mannix@childrens.harvard.edu; Tel: (617) 355-6624; Fax (617) 730-0335.

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Introduction

Traumatic Brain Injury (TBI) is a devastating health problem associated with a rehabilitative cost of 60 billion dollars annually in the United States.²⁰ No single pharmacologic agent has been definitively shown to significantly improve secondary injury after human TBI. Recently, there has been increasing interest in the potential role of progesterone as a promising therapeutic candidate.¹³

Progesterone is a steroid hormone involved in the female menstrual cycle, pregnancy, and embryogenesis. The neuroprotective potential of progesterone was initially noted when experimental models demonstrated that female rats had improved functional outcomes after TBI compared to male rats.¹⁹ Animal studies suggest that progesterone modulates excitotoxicity,^{3,5} down regulates the inflammatory cascade,^{2,11} reconstitutes the bloodbrain barrier,⁶ and reduces cerebral edema.^{18,24} A recent phase II randomized-controlled trial demonstrated some modest benefits in survival and functional outcomes for adults of both genders who received progesterone after non-penetrating TBI.²⁵

Despite the promise of this recent clinical investigation, it is unclear whether progesterone is an appropriate therapeutic agent across the spectrum of age. Prior studies have suggested that the effectiveness of at least some therapies may depend on age.^{14,17} Moreover, some experimental models have suggested age-dependent differences in cerebral edema,¹⁰ inflammatory response,^{1,4} blood-brain permeability and excitotoxicity²³ in immature compared to adult animals after brain injury. Despite the promise of progesterone in adult models of brain injury, one recent study suggested a harmful effect of progesterone after hypoxic-ischemic injury in immature rats, whereby progesterone worsened neuropathological injury.²¹ Understanding whether or not progesterone has protective or harmful effects in children is important because the greatest public health burden of TBI occurs in this setting. Here, we tested the hypothesis that, following controlled cortical impact (CCI), treatment with progesterone will improve functional and histopathological outcomes in adolescent mice. We included both male and female mice, as the vast majority of preclinical studies in progesterone have included only male and/or reproductively senescent female mice.

Methods

CCI Injury and Progesterone Treatment

All experiments were approved by the Boston Children's Hospital Institutional Review Board and complied with the NIH Guide for the Care and Use of Laboratory Animals. A total of 18 male and 18 female adolescent (4 week old) C57Bl/6J mice were used (Jackson Laboratories, Bar Harbor Maine). Mice were randomized to treatment with progesterone in cyclodextrin vehicle (8mg/kg ip n = 32/group) or vehicle alone (0.215 mmol cyclodextrin, i.p., n = 26/group) for a total volume 12.5 ul based on prior studies in mice.^{7,9,12} Mice were injected at 2 minutes post injury, 6 hours post injury, and daily for 4 days after injury, for a total of 6 doses. For all experiments, investigators were blinded to treatment groups.

The mouse CCI model was used as previously described.¹⁵ Mice were randomized to CCI injury (n=34 total, n=10 males progesterone treated, n= 7 males vehicle treated, n= 10 females progesterone treated, n=7 females vehicle treated) or sham injury (n=24, n= 6 males progesterone treated, n= 6 males vehicle treated, n= 6 females progesterone treated, n= 6 females vehicle treated). Mice were anesthetized using 3% isoflurane in a 70:30 mixture of oxygen and placed in a stereotactic frame. A 5-mm craniotomy was performed over the left parietotemporal cortex and the bone flap was removed. Mice underwent CCI using a pneumatic cylinder with a 3-mm flat-tip impounder, velocity 6 m/s, and impact depth of 0.6 mm. Sham injured mice underwent anesthesia and craniotomy only. To reduce variability in injury level attributable to environment, all mice were injured by the same experimenter who was blinded to group assignment.

Motor and Memory Testing

Gross vestibulomotor function was assessed using the wire grip test¹⁵ days 1–8 after injury. The test consisted of placing the mouse on a wire suspended between two poles and grading the degree of attachment and movement of the mouse. Scores were as follows: 0 was given to a mouse which fell from the wire within 30 s; 1 point for unilateral grasp of either upper or lower extremities, 2 points for midline grasp of both upper and lower extremities but not the tail; 3 points for midline grasp of all extremities plus the tail; 4 points for movement along the wire after achieving a score of 3; and 5 points for a score of 4 plus climbing down the pole within 60 s.

Spatial memory performance was assessed 4–5 weeks after injury using the Morris water maze (MWM).^{15,16} A white pool (83 cm diameter, 60 cm deep) was filled with water to 29 cm depth. Several highly visible intra- and extra-maze cues that remained constant throughout the trials were located in and around the pool. Water temperature was maintained approximately 24°C. The goal platform (a round, clear, Plexiglas platform 10 cm in diameter) was positioned 1 cm below the surface of the water. Each mouse was subjected to a maximum of two trials per day. Each trial consisted of four subtrials in which mice started at each of four locations (north, south, east, or west). For each subtrial, mice were placed in the pool in the selected quadrant facing the wall. Mice were given a maximum of 90 s to find and rest upon the submerged platform. If the mouse did not rest on the platform by 90 s, it was gently placed there for 10 s by the experimenter. For probe trials, mice were placed in the pool with the platform removed. The time that the animal swam in the target quadrant was recorded (maximum 60 s). For visible platform trials, the goal platform was marked by red tape and placed ½ cm above the water level. Performance in the MWM was quantitated by latency to the platform for hidden trials (90 s maximum) or latency in the target quadrant for probe trials (60 s maximum).

Lesion Volume

After MWM testing, mice were sacrificed and brains removed and assessed for lesion volume as we have previously reported.¹⁵ Briefly, coronal brain sections (12 µm) were cut every 0.3–0.5 mm from the anterior to the posterior brain, mounted on poly-L-lysine-coated slides, and stained with hematoxylin (Surgipath). Using the Measure Tool on Image J (Version 1.44), a blinded investigator calculated hemispheric brain volume. Lesion volume

was obtained by subtracting the volume of brain tissue remaining in the left (injured) hemisphere from that of the right (uninjured) hemisphere and expressed as percent volume lost.

Statistical Analyses

All statistical analyses were performed using Stata 11.2 (StataCorp, College Station, TX). Data are mean $\pm$ SEM or median (interquartile range, IQR). Motor data, which yield ordinal responses, were analyzed with ordinal logistic regression with clustering to account for repeated measures. MWM data, which are continuous, were analyzed by linear regression with clustering to account for repeated measures. For all analyses, an interaction term was used to evaluate whether or not the effect of progesterone treatment was different in male vs. female mice. Volumetric data were analyzed using rank sum test or analysis of variance as appropriate. For all tests, $p < 0.05$ was considered significant. Based on our prior studies, we estimated we would need 8–10 mice/group to detect a 30% difference in means with a SD 30% given an alpha of 0.05 and a power of 0.80.

Results

All mice survived CCI. There were no significant differences in baseline motor performance between sham male progesterone-treated versus vehicle-treated mice, or between sham female progesterone-treated versus sham vehicle-treated mice (all groups, $n=6/\text{group}$). Injured mice ($n=34$) performed worse than sham mice ($n=24$, $p<0.001$). Combining male and female CCI injured mice, there was no difference in motor performance between progesterone and vehicle treated mice ($p=0.9$). However, the effect of progesterone treatment after CCI was different for gender ($p = 0.001$ for the interaction term). For CCI injured male mice, wire grip scores were improved after progesterone treatment ($n=7-10/\text{group}$, $p < 0.001$, Figure 1a), but were significantly worse for CCI injured female mice treated with progesterone ($n=7-10/\text{group}$, $p = 0.001$, Figure 1b).

For MWM, all injured mice demonstrated time-dependent improvements in latency to the hidden platform, indicating the ability to learn the MWM paradigm after CCI ($p < 0.001$ for time for all groups). There was no significant difference in hidden platform trial performance between CCI injured progesterone-treated versus CCI injured vehicle-treated male mice, or between sham progesterone-treated versus sham vehicle-treated male mice (Figure 2a). Likewise, there was no significant difference in hidden platform trial performance between female CCI injured progesterone-treated versus vehicle-treated mice, or between sham-injured progesterone-treated versus vehicle treated female mice (Figure 2b). There were no significant group differences in visual platform or probe trial performance.

At 5 weeks after CCI, injured mice showed well-demarcated cavitory lesions involving the cortex and hippocampus of injured hemispheres (Figure 2c). There were no statistical differences in lesion volume between CCI injured male progesterone-treated versus vehicle-treated mice ($24\% \pm 2\%$ vs. $27\% \pm 1\%$, $n=7-10/\text{group}$, $p = 0.2$) or in CCI injured progesterone-treated vs. vehicle-treated female mice ($27\% \pm 1\%$ vs. $23\% \pm 2\%$, $n=7-10/\text{group}$, $p = 0.1$).

Discussion

We found gender specific differences in vestibulomotor outcome after progesterone treatment in a pediatric model of TBI. Prior studies have offered contradictory results of progesterone treatment after brain injury in immature animals.^{21,22} While this study was initially designed to evaluate whether or not progesterone was a safe and effective therapy for adolescent mice after CCI, we unexpectedly found gender differences in 4 week old injured mice in response to progesterone treatment. Though numerous prior studies have suggested a beneficial effect of progesterone in the setting of TBI, the vast majority of these studies have used adult males, adult ovariectomized females or reproductively senescent females.⁹ While progesterone has been shown to protect aging female mice after cerebral ischemia,⁸ whether or not progesterone treatment is beneficial for immature female mice after TBI has not been established. Our results suggest that only male adolescent mice receive a beneficial effect from progesterone after CCI, but treatment of female adolescent mice might be detrimental. These findings may be relevant to current and future clinical trials of progesterone after TBI. Though the ProTECT II trial, a phase II trial of progesterone after TBI, showed no detrimental effect of treatment, it was not powered to discern age and gender specific differences in outcomes. Moreover, the current phase III trial, which does not include children < 18 years, may also not be able to address gender specific differences given the male predominant epidemiology of TBI. Future studies will need to address the mechanism of a potential gender dependent effect of treatment after injury, which could be due to numerous factors including age and gender specific differences in metabolism (peripheral and central), CNS progesterone receptor expression, progesterone-mediated GABAergic neuroexcitability, or post-injury apoptotic responses.

This study has several limitations. First, it is possible that the dose-response curve of progesterone is u-shaped, and that progesterone would indeed have a protective effect in female adolescent mice at other doses and/or dosing regimens. However, demonstrating sex-dependent differences in efficacy versus toxicity has important implications for clinical studies evaluating the effect of progesterone in the setting of TBI. Second, we did not evaluate serum or tissue levels of progesterone after dosing, and it is certainly possible that some of the effects represent differences in peripheral and/or central metabolism. However, we derived our dosing from a prior mouse model of brain injury, which demonstrated a protective effect of progesterone after cerebral ischemia in male mice.⁷ Third, we did not account for the estrous status of female mice in our study, and the gender specific effects associated with progesterone may therefore not be completely attributable to progesterone alone. Fourth, we did not evaluate serum or tissue progesterone levels prior to progesterone dosing, and there may have been considerable heterogeneity in sexual maturation and hormonal status in these young mice, as there would similarly be in a clinical population of adolescents. Fifth, our study was small, and though we found a significant effect of treatment on motor outcomes, we may have been underpowered to detect differences in Morris Water Maze or histopathological outcomes. Finally, we tested a limited set of behavioral and pathological outcomes, and more extensive testing is warranted to evaluate the post treatment motor deficits described in this study.

Conclusion

Further characterization of the effect of progesterone on female adolescent mice is needed in larger cohorts prior to any attempt to translate these results clinically and the molecular mechanism of this effect should be elaborated. Nonetheless, this study indicates that progesterone may have gender specific effects on adolescent mice after CCI, a finding that could have significant relevance to clinical trials in children.

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Figure 1.

Wire grip testing after controlled cortical impact (CCI) demonstrates a protective effect in progesterone treated male mice, but a deleterious effect in progesterone treated female mice.

A) Compared to vehicle treated mice, progesterone treated adolescent male mice showed improved vestibulomotor function up to 8 days after injury ($p < 0.001$). B) Progesterone-treated adolescent female mice had worse performance on wire grip compared to vehicle treated adolescent female mice ($p = 0.001$).

Figure 2.

No differences in Morris water maze testing (MWM) and lesion volume after CCI and treatment. A) Compared to vehicle treated injured male mice, injured progesterone treated adolescent male mice showed no difference in hidden or visual platform trial performance ($p=0.6$). B) Progesterone-treated injured adolescent female mice had similar performance on MWM compared to vehicle treated injured adolescent female mice ($p=0.6$). C) On lesion volume analysis, there were no differences in percent tissue lost in progesterone versus vehicle treated injured adolescent male mice or progesterone versus vehicle treated injured adolescent female mice (representative photos shown here).