Sex differences in the effect of progesterone after controlled cortical impact in adolescent mice: a preliminary study

Laboratory investigation

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Object. While progesterone has been well studied in experimental models of adult traumatic brain injury (TBI), it has not been evaluated in pediatric models. The study of promising interventions in pediatric TBI is important because children have the highest public health burden of such injuries. Therapies that are beneficial in adults may not necessarily be effective in the pediatric population. The purpose of this study was to evaluate whether progesterone treatment improves outcomes in an experimental model of pediatric TBI.

Methods. The authors determined whether progesterone administered after controlled cortical impact (CCI) improves functional and histopathological outcomes in 4-week-old mice. Both male and female mice (58 mice total) were included in this study, as the majority of prior studies have used only male and/or reproductively senescent females. Mice were randomized to treatment with progesterone or vehicle and to CCI injury or sham injury. Motor (wire grip test) and memory (Morris water maze) testing were performed to determine the effect of progesterone on TBI. Lesion volume was also assessed.

Results. Compared with their vehicle-treated counterparts, the progesterone-treated CCI-injured male mice had improved motor performance (p < 0.001). In contrast, progesterone-treated CCI-injured female mice had a worse performance than their vehicle-treated counterparts (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 sex-specific effect of progesterone treatment after CCI in adolescent mice and could inform clinical trials in children.

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Key Words • traumatic brain injury • progesterone • adolescent • sex differences • neuroprotection

TRAUMATIC brain injury (TBI) is a devastating health problem associated with a rehabilitative cost of $60 billion annually in the United States.20 No single pharmacological agent has been definitively shown to significantly improve secondary injury after TBI. Recently, there has been increasing interest in progesterone as a promising therapeutic agent.13

Progesterone is a steroid hormone involved in the female menstrual cycle, pregnancy, and embryogenesis. Its neuroprotective potential was initially noted when experimental models demonstrated that female rats had improved functional outcomes after TBI as compared with those in male rats.19 Animal studies have suggested that progesterone modulates excitotoxicity,3,5 downregulates the inflammatory cascade,2,11 reconstitutes the blood-brain barrier,4 and reduces cerebral edema18,24. A recent phase II randomized controlled trial demonstrated some modest benefits in survival and functional outcomes for adults of both sexes who had received progesterone after nonpenetrating TBI.25

Despite the positive findings in 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,\textsuperscript{10} inflammatory response,\textsuperscript{1,4} blood-brain barrier permeability, and excitotoxicity\textsuperscript{23} in immature compared with adult animals after brain injury. Despite progesterone’s promise in adult models of brain injury, one recent study suggested a harmful effect of progesterone after hypoxic-ischemic injury in immature rats, whereby the hormone worsened neuropathological injury.\textsuperscript{23} Understanding whether progesterone has protective or harmful effects in children is important because the greatest public health burden of TBI occurs in this population. Here, we tested the hypothesis that, following controlled cortical impact (CCI), treatment with progesterone would improve functional and histopathological outcomes in adolescent mice. We included both male and female mice in the study, as most preclinical studies of progesterone have included only male and/or reproducitively senescent female mice.

Methods

Controlled Cortical Impact 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. Twenty-nine male and 29 female adolescent (4 weeks old) C57Bl/6 J mice were used (Jackson Laboratories). Mice were randomized to treatment with progesterone in cyclodextrin vehicle (8 mg/kg, intraperitoneally, 32 mice total: 16 males, 16 females) or with vehicle alone (0.215 mmol cyclodextrin, intraperitoneally, 26 mice total: 13 males, 13 females) for a total injected volume of 12.5 µl based on prior studies in mice.\textsuperscript{7,9,12} The mice were injected at 2 minutes postinjury, 6 hours postinjury, and daily every day after injury for 4 days, for a total of 6 doses. For all experiments, investigators were blinded to the treatment groups.

The mouse CCI model has been previously described.\textsuperscript{15} In brief, mice were anesthetized using 3% isoflurane in a 70:30 mixture of nitrogen/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/sec, and impact depth of 0.6 mm. Sham-injured mice were anesthetized and underwent craniotomy only. To reduce the variability in injury level attributable to environment, the same experimenter, who was blinded to group assignment, delivered the injury to all mice. Mice were randomized to CCI injury (34 mice total: 17 males [10 progesterone treated, 7 vehicle treated], 17 females [10 progesterone treated, 7 vehicle treated]) or to sham injury (24 mice total: 12 males [6 progesterone treated, 6 vehicle treated], 12 females [6 progesterone treated, 6 vehicle treated]).

Motor and Memory Testing

Gross vestibulomotor function was assessed using the wire grip test\textsuperscript{15} on Days 1–8 after injury. The test involved placing the mouse on a wire suspended between 2 poles and grading the degree of attachment and movement of the mouse. Scores were as follows: 0 points, fall from the wire within 30 seconds; 1 point, unilateral grasp by either upper or lower extremity, 2 points, midline grasp by both upper and lower extremities but not the tail; 3 points, midline grasp by all extremities plus the tail; 4 points, movement along the wire after achieving a score of 3; and 5 points, a score of 4 plus climbing down the pole within 60 seconds.

Spatial memory performance was assessed 4–5 weeks after injury using the Morris water maze (MWM).\textsuperscript{15,16} A white pool (83 cm in diameter, 60 cm deep) was filled with water to a depth of 29 cm. 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 at 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 2 trials per day. Each trial consisted of 4 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 seconds to find and rest upon the submerged platform. If the mouse did not rest on the platform by 90 seconds, the experimenter gently placed it there for 10 seconds. For probe trials, mice were placed in the pool with the platform removed. The amount of time that the animal swam in the target quadrant was recorded (maximum 60 seconds). For visible platform trials, the goal platform was marked by red tape and placed 1/2 cm above the water level. Performance in the MWM was quantitated by latency to the platform for hidden trials (90 seconds maximum) or latency in the target quadrant for probe trials (60 seconds maximum).

Lesion Volume

After MWM testing, mice were euthanized, and their brains were removed and assessed for lesion volume, as previously described.\textsuperscript{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 the percentage volume lost.

Statistical Analyses

All statistical analyses were performed using Stata 11.2 (StataCorp). Data are presented as the mean ± standard error of the mean or median (interquartile range [IQR]). Motor data, which yield ordinal responses, were analyzed using ordinal logistic regression with clustering to account for repeated measures. Morris water maze data, which are continuous, were analyzed using linear regression with clustering to account for repeated measures. For all analyses, an interaction term was used to
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evaluate whether the effect of progesterone treatment was different in male versus female mice. Volumetric data were analyzed using the rank-sum test or ANOVA as appropriate. For all tests, p < 0.05 was considered significant. Given our prior studies, we estimated that we would need 8–10 mice/group to detect a 30% difference in means with an SD of 30% given an alpha of 0.05 and a power of 0.80.

Results

All mice survived CCI. There was no significant difference in baseline motor performance between progesterone- and vehicle-treated male sham-injured mice, nor between progesterone- and vehicle-treated female sham-injured mice (6 mice/group). Overall, injured mice (34) performed less well than the sham-injured mice (24, p < 0.001). Among all the CCI-injured mice, there was no difference in motor performance between the progesterone- and vehicle-treated mice (p = 0.9). However, the effect of progesterone treatment after CCI was different for each sex (p = 0.001 for the interaction term). For CCI-injured male mice, wire grip scores were improved after progesterone treatment (7–10 mice/group, p < 0.001; Fig. 1) but were significantly worse for CCI-injured female mice treated with progesterone (7–10 mice/group, p = 0.001).

For the MWM, all injured mice demonstrated 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 performance on the hidden platform trial between progesterone- and vehicle-treated male CCI-injured mice, nor between progesterone- and vehicle-treated male sham-injured mice (Fig. 2A). Likewise, there was no significant difference in performance on the hidden platform trial between progesterone- and vehicle-treated female CCI-injured mice, nor between progesterone- and vehicle-treated female sham-injured mice (Fig. 2B). There were no significant group differences in the visible platform or probe trial performance.

At 5 weeks after CCI, injured mice showed well-demarcated cavitary lesions involving the cortex and hippocampus of injured hemispheres (Fig. 2C). There were no statistical differences in lesion volume between progesterone- and vehicle-treated male CCI-injured mice (24% ± 2% vs 27% ± 1%, 7–10 mice/group, p = 0.2), nor between progesterone- and vehicle-treated female CCI-injured mice (27% ± 1% vs 23% ± 2%, 7–10 mice/group, p = 0.1).

Discussion

We found sex-specific differences in vestibulomotor outcome after progesterone treatment in a pediatric model of TBI. Prior studies have offered contradictory results on progesterone treatment after brain injury in immature animals.1,2 While the present study was initially designed to evaluate whether progesterone was a safe and effective therapy for adolescent mice after CCI, we unexpectedly found sex differences in 4-week-old injured mice in response to progesterone treatment. Though numerous studies have suggested a beneficial effect of progesterone in the setting of TBI, the majority of these studies have used adult males, adult ovariectomized females, or reproductively senescent females.3 While progesterone has been shown to protect aging female mice after cerebral ischemia,4 whether 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 and that the treatment of female adolescent mice may be detrimental. These findings may be relevant to current and future clinical trials of progesterone after TBI. Although 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 sex-specific differences in outcomes. Moreover, the current phase III trial, which does not include children (age < 18 years), may not be able to address sex-specific differences given the male predominance in TBI. Future studies will need to address the mechanism of a potential sex-dependent effect of treatment after injury, which could be attributable to numerous factors including age- and sex-specific differences in metabolism (peripheral and central), CNS progesterone receptor expression, progesterone-mediated GABAergic neuroexcitability, or postinjury 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 adolescent female 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. Third, we did not account for the estrous status of female mice in our study; thus, the sex-specific effects associated with progesterone may 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, our study may have been underpowered to detect differences in MWM testing or histopathological outcomes. Finally, we tested a limited set of behavioral and pathological outcomes, and more extensive testing is warranted to evaluate the posttreatment motor deficits described in this study.

Conclusions

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

Disclosure

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