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Progesterone exerts neuroprotective effects and improves long-term neurologic outcome after intracerebral hemorrhage in middle-aged mice

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ABSTRACT

In this study, we examined the effect of progesterone on histopathologic and functional outcomes of intracerebral hemorrhage (ICH) in 10- to 12-month-old mice. Progesterone or vehicle was administered by intraperitoneal injection 1 hour after collagenase-induced ICH and then by subcutaneous injections at 6, 24, and 48 hours. Oxidative and nitrosative stress were assayed at 12 hours post-ICH. Injury markers were examined on day 1, and lesion was examined on day 3. Neurologic deficits were examined for 28 days. Progesterone posttreatment reduced lesion volume, brain swelling, edema, and cell degeneration and improved long-term neurologic function. These protective effects were associated with reductions in protein carbonyl formation, protein nitrosylation, and matrix metalloproteinase-9 activity and attenuated cellular and molecular inflammatory responses. Progesterone also reduced vascular endothelial growth factor expression, increased neuronal-specific Na^+/K^+ ATPase α3 subunit expression, and reduced protein kinase C–dependent Na^+/K^+ ATPase phosphorylation. Furthermore, progesterone reduced glial scar thickness, myelin loss, brain atrophy, and residual injury volume on day 28 after ICH. With multiple brain targets, progesterone warrants further investigation for its potential use in ICH therapy.

1. Introduction

Intracerebral hemorrhage (ICH), a common and devastating subtype of stroke, accounts for 15%–20% of all strokes and affects >2 million people worldwide each year (Adeoye and Broderick, 2010; Poon et al., 2015; Wang, 2010). With limited therapeutic options, ICH is associated with high morbidity and poor prognosis. It causes perihematomal edema, elevations in intracranial pressure, and neurologic deficits (Keep et al., 2014). Hematoma formation, expansion, and mass effect cause the primary damage, whereas inflammatory response and oxidative stress contribute to the progression of secondary injury (Wang, 2010; Wu et al., 2010). Because outcomes are poor, it is worth exploring approaches that can ameliorate the detrimental effects of neuroinflammation and improve functional recovery after ICH.

We and others have revealed that inflammatory mediators cyclooxygenase (COX)-1, COX-2, prostaglandin receptors, high-mobility group box 1 (HMGB1), and interleukin 1β (IL-1β) may play major roles in ICH-induced secondary injury (Han et al., 2015; Mracsko and Veltkamp, 2014; Wang, 2010; Wu et al., 2011b; Wu et al., 2015, 2016). A recent clinical trial reported that patients treated in the acute stage of ICH with celecoxib, a selective COX-2 inhibitor, had a smaller expansion of perihematomal edema than controls (Lee et al., 2013). Increased release of HMGB1 and IL-1β after ICH may boost inflammatory reaction in the early stage by promoting secretion of other chemotactic factors and adhesion molecules from the vascular endothelium. These factors can lead to the activation of glial cells and early infiltration of neutrophils and macrophages to the injury lesion (Li et al., 2015; Wu et al., 2015). Infiltrating polymorphonuclear neutrophils that accumulate within and around the lesion release toxic substances such as matrix metalloproteinase (MMP)-2 and MMP-9 and promote further inflammatory damage (Wang, 2010; Wang and Dore, 2007b; Xue and

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Yong, 2008). Inflammatory response also enhances the release of reactive oxygen species (ROS) that lead to cell death and brain tissue damage after ICH (Wang, 2010; Wang and Dore, 2007b). In addition to activation of MMP-2 and -9 released from inflammatory cells, increased expression of vascular endothelial growth factor (VEGF) and decreased activity of Na^+/K^+ ATPase (NKA) also play a role in brain edema formation after stroke (Keep et al., 2014; Won et al., 2014; Zan et al., 2014). Therefore, modulation of inflammatory response after ICH could offer a promising therapeutic approach to ICH.

Progesterone, primarily a sex hormone, has been shown to exert neuroprotective effects by alleviating inflammatory response and improving neurologic function after ischemic stroke (Jiang et al., 2009, 2011, 2016; Won et al., 2015). To our knowledge, no study has reported a therapeutic effect of progesterone after ICH in middle-aged mice. Therefore, we investigated whether progesterone administered after ICH provides neuroprotection and improves long-term neurologic outcome for ICH. Because a 12-month-old mouse is equivalent to a person of 58 years (www.age-converter.com/mouse-age-calculator.html), middle age in mice equates to 10–15 months (Dutta and Sengupta, 2015). Therefore, to enhance the clinical relevance, we used 10- to 12-month-old mice, as ICH occurs more often in middle-aged and elderly individuals. For early outcomes of ICH, we evaluated inflammatory response, oxidative stress, neuronal death, brain injury volume, brain swelling, and brain edema in the acute phase after ICH; for long-term outcomes of ICH, we evaluated astrogliosis (glial scar thickness), myelin loss, brain atrophy, and residual lesion volume on day 28. Neurologic deficits were assessed on days 1, 3, 7, 14, 21, and 28 post-ICH.

2. Materials and methods

2.1. Animals, ICH model, and treatment regimen

All animal procedures were performed in accordance with the National Institutes of Health and institutional guidelines for animal research under a protocol approved by the Animal Care and Use Committee of Johns Hopkins University. One hundred thirty middle-aged male C57BL/6 mice weighing 25–35 g (10–12 months old) were used and were given free access to food and water throughout the study. Animal experiments were reported in accordance with the ARRIVE guidelines.

The procedure for inducing ICH by collagenase injection in mice has been established in our laboratory (Chang et al., 2014; Zhao et al., 2015). Briefly, mice were anesthetized by isoflurane (3.0% for induction and 1.0% for maintenance) and ventilated with oxygen-enriched air (20%:80%) via a nose cone. To induce hemorrhage, we injected the left caudate putamen of mice with collagenase VII-S (0.075 U in 0.5 μL saline; Sigma-Aldrich, St Louis, MO, USA) at the following stereotactic coordinates: 0.8 mm anterior and 2.0 mm lateral of the bregma and 2.9 mm in depth. Collagenase was delivered >5 minutes, and the needle was left in place for an additional 10 minutes to prevent any reflux. Rectal temperature was maintained at 37.0 ± 0.5 °C throughout the experimental and recovery periods. The rectal temperature and percent change in body weight of each mouse were recorded as previously described (Wu et al., 2015).

Computer-generated random numbers were used for randomization of mice into 3 study groups: sham-operated group, vehicle-treated ICH group, and progesterone-treated ICH group. Progesterone (8 mg/kg; Sigma-Aldrich) or vehicle (22.5% 2-hydroxypropylcyclodextrin; Sigma-Aldrich) was injected intraperitoneally at 1 hour after surgery and subcutaneously at 6, 24, and 48 hours (Wali et al., 2014). Investigators blinded to the treatment groups evaluated outcomes in all mice and performed data analysis.

2.2. Brain lesion volume, swelling, and atrophy

Mice (n = 10–11 per group) were euthanized after neurologic examination on day 3 or 28 post-ICH. The entire brain of each mouse was cut with a cryostat into 50-μm sections at 10 rostral-caudal levels that were spaced 360 μm apart. Sections were stained with Luxol fast blue (LFB, for myelin) and Cresyl Violet (for neurons) before being quantified for gray- and white matter injuries with SigmaScan Pro software (version 5.0.0 for Windows; Systat, San Jose, CA, USA). The lesion volume in cubic millimeters was calculated by multiplying the section thickness by the damaged area, as determined by the lack of specific staining (Wang et al., 2003).

Brain swelling (n = 10–11 mice/group) was quantified by calculating the percentage of hemispheric enlargement on day 3 after ICH (Wu et al., 2011a). Hemisphere enlargement (%) was expressed as ([ipsilateral hemisphere volume − contralateral hemisphere volume]/contralateral hemisphere volume) × 100%.

Brain atrophy (tissue loss, n = 12 mice per group) was quantified on day 28 after ICH according to the formula: (contralateral hemisphere volume − ipsilateral hemisphere volume)/contralateral hemisphere volume ×100% (Wu et al., 2015).

2.3. Brain water content

Mice (n = 6 per group) were anesthetized and decapitated on day 3 after ICH as previously described (Jiang et al., 2009; Zhu et al., 2014) for determination of brain water content. Briefly, cerebral tissue was divided into 2 hemispheres. We immediately dissected and weighed the ipsilateral and contralateral striatum and cerebellum (that served as an internal control) with an electronic analytical balance to obtain the wet weight. Then, after brain samples were dried at 100 °C in an electric blast drying oven for 24 hours, we obtained the dry weight. The percentage of brain water content was calculated as (wet weight − dry weight)/wet weight × 100%.

2.4. Neurologic deficit assessment

The modified neurologic severity score was used to assess the neurologic deficits of mice on days 1, 3, 7, 14, and 28 after ICH or sham surgery (n = 12 mice per group) (Jiang et al., 2013; Wang and Dore, 2007a; Zhao et al., 2015). The modified neurologic severity score comprises body symmetry, gait, climbing, circling behavior, front limb symmetry, and compulsory circling. Neurologic deficit was graded on a scale of 0–4 for each test, establishing a maximum deficit score of 24.

2.5. Gelatin gel zymography

The MMP-2 (gelatinase A, 72 kD) and MMP-9 (gelatinase B, 98 kD) proteolytic activities in hemorrhagic brain were measured with gelatin gel zymography on day 1 post-ICH as previously described (Wang and Tsirka, 2005). Briefly, the brains were collected, and the hemorrhagic hemispheres were sonicated in ice-cold homogenization buffer (20 mM Tris, 1 mM ethylene glycol tetraacetic acid, 1 mM EDTA, 10% sucrose, pH 7.4) containing protease inhibitors. Protein samples were obtained by centrifugation at 1000 rpm for 10 minutes at 4 °C. Samples were loaded onto 10% Tris-tricine gels with 0.1% gelatin as a substrate and separated by electrophoresis. Next, the gel was renatured and incubated with development buffer at 37 °C for 48 hours. After development, the gel was stained with 0.5% wt/vol Coomassie blue R-250 for 2 hours and then destained appropriately to be photographed. MMP-2/9 activity was measured by optical density and quantified as fold increase compared with