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Sex-Specific Effects of Progesterone on Early Outcome of Intracerebral Hemorrhage

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Key Words

Intracerebral hemorrhage · Stroke · Progesterone · Sex difference · Cerebral edema · Neuroinflammation · Cytokines · Rat · Neurobehavioral recovery · Magnetic resonance imaging

Abstract

Background: Preclinical evidence suggests that progesterone improves recovery after intracerebral hemorrhage (ICH); however, gonadal hormones have sex-specific effects. Therefore, an experimental model of ICH was used to assess recovery after progesterone administration in male and female rats. ***Methods:*** ICH was induced in male and female Wistar rats via stereotactic intrastriatal injection of clostridial collagenase (0.5 U). Animals were randomized to receive vehicle or 8 mg/kg progesterone intraperitoneally at 2 h, then subcutaneously at 5, 24, 48, and 72 h after injury. Outcomes included relevant physiology during the first 3 h, hemorrhage and edema evolution over the first 24 h, proinflammatory transcription factor and cytokine regulation at 24 h, rotarod latency and neuroseverity score over the first 7 days, and microglial activation/macrophage recruitment at 7 days after injury. ***Results:*** Rotarod latency ($p = 0.001$) and neuroseverity score ($p = 0.01$) were improved in progesterone-treated males, but worsened in progesterone-treated females ($p = 0.028$ and $p = 0.008$, respectively). Progesterone decreased cerebral edema ($p = 0.04$), microglial activation/macrophage recruitment ($p < 0.001$), and proinflammatory transcription factor phosphorylated nuclear factor-κB p65 expression ($p = 0.0038$) in males but not females, independent of tumor necrosis factor-α, interleukin-6, and toll-like receptor-4 expression. Cerebral perfusion was increased in progesterone-treated males at 4 h ($p = 0.043$) but not 24 h after injury. Hemorrhage volume, arterial blood gases, glucose, and systolic blood pressure were not affected. ***Conclusions:*** Progesterone administration improved early neurobehavioral recovery and decreased secondary neuroinflammation after ICH in male rats. Paradoxically, progesterone worsened neurobehavioral recovery and did not modify neuroinflammation in female rats. Future work should isolate mechanisms of sex-specific progesterone effects after ICH.

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Introduction

Intracerebral hemorrhage (ICH) accounts for 10–15% of all first-time strokes in the United States [1]. It is associated with 1-month mortality of 40% and significant morbidity when compared to other stroke subtypes [2, 3]. A number of studies have examined the epidemiology of ICH, but the effect of sex on the incidence and mortality is not well characterized. Research findings from different parts of the world have demonstrated that women may have a lower incidence of ICH [4–6], less perihematomal edema, and lower mortality [7–9]. These findings raise the important question of whether endogenous gonadal hormones are involved in protection against damaging ICH effects.

Emerging data from preclinical studies demonstrate that progesterone plays a neuroprotective role in a wide range of neurological diseases. In traumatic brain injury (TBI), progesterone prevented neuronal loss and improved functional recovery through suppressing neuroinflammation [10–13], reducing cerebral edema [14–16], attenuating oxidative stress [17], preserving mitochondrial function [18, 19], minimizing lipid peroxidation [20], and preventing cellular apoptosis [21, 22]. In ischemic stroke, progesterone reduced infarction size [23, 24], suppressed neuroinflammation [25–27], minimized hemorrhagic transformation [28, 29], decreased blood-brain barrier permeability [30], and improved outcome [31–34]. Progesterone showed similar beneficial effects in a rat model of subarachnoid hemorrhage (SAH) through attenuating vasospasm [35], preventing apoptosis [36], and reducing oxidative stress [37, 38]. Progesterone also improved functional recovery in other neuroinflammatory diseases, including demyelinating diseases [39, 40] and diabetic neuropathy [41].

In spite of this wealth of preclinical data in other neurological diseases, little is known regarding the role of progesterone in ICH, a mechanistically different disease from TBI, ischemic stroke, and SAH. Chen et al. [42] showed that exogenous progesterone administration decreased brain edema and improved motor function in male rats at 24 h after intrastriatal autologous blood injection. Although promising, the potential efficacy of progesterone after ICH remains unclear and must be fully explored before its prospective therapeutic value can be tested in clinical trials. To this end and to closely mimic the human disease, we employed a rat model of collagenous-induced intrastriatal ICH for its consistent and reproducible hematoma with expansion over the initial 24 h [43]. Sex differences in the response to progesterone treatment after ICH were investigated.

Methods

All procedures were designed to minimize animal discomfort and numbers, conformed with international guidelines on the use of animals, and were approved by the Duke University Institutional Animal Care and Use Committee. All animals were randomized to concealed treatment groups before injury. One investigator (B.L.) concealed treatment assignments. Blinded investigators (J.T.H. and H.S.) performed all surgical procedures and recorded outcomes. Animals were excluded from outcome analyses only if they died during the surgical procedure (n = 2, both in neurobehavior cohort: 1 progesterone- and 1 vehicle-treated).

Experimental Groups and Randomization

Adult (250–300 g) male and female Wistar rats, male spontaneously hypertensive rats (SHRs), and male Wistar-Kyoto rats (WKRs) were used in these experiments (Charles River Laboratory, Wilmington, Mass., USA). Rats aged 8–16 weeks were selected to normalize body/brain weight between sexes, and approximate age to young adult humans [44]. Before inducing ICH, rats were number-coded and randomized to receive vehicle (sesame oil) or progesterone (8 mg/kg) intraperitoneally at 2 h and subcutaneously at 5, 24, 48, and 72 h after injury. Initial intraperitoneal injection ensures rapid absorption, while the subcutaneous route allows for sustained absorption. Progesterone dosing and outcome measurements were chosen based on prior work with progesterone in TBI [42]. Sham animals demonstrated recovery patterns exactly like uninjured animals, consistent with previous work [45]. The cohorts were arranged as follows. Cohort 1: Neurobehavioral recovery over the first 7 days after ICH in male and female Wistar rats treated with progesterone or vehicle. After 7 days, rats were euthanized for immunohistochemistry and stereological identification of microglial activation/macrophage recruitment (n = 10 rats/group). Cohort 2: Hemorrhage volume by histology at 24 h after ICH in male and female Wistar rats treated with progesterone or vehicle after ICH (n = 5 rats/group). Cohort 3: Brain water content at 24 h after ICH in males (n = 8 Wistar rats/group) and females (n = 5 Wistar rats/group) treated with progesterone or vehicle after ICH. Cohort 4: Proinflammatory cytokines at 24 h after ICH in male and female Wistar rats treated with progesterone or vehicle after ICH (n = 5 rats/group). Cohort 5: Brain magnetic resonance imaging (MRI) and physiological measurement of blood pressure, arterial gas concentrations, and hemispheric cerebral blood flow in male WKRs and SHRs (to mimic the common comorbid condition of high blood pressure) treated with progesterone or vehicle over the first 24 h after ICH (n = 3 rats/group).

Vaginal Smear

Vaginal washes were performed to determine the stage of the estrous cycle [46]. Injury was induced during the diestrous period since the endogenous serum progesterone concentration is highest at this stage of the cycle without increasing the estrogen concentration [47, 48].

Anesthesia

All animals were anesthetized in a closed chamber with 5% isoflurane in 30% O_2/balance N_2. The trachea was intubated with a 16-gauge Insyte-W intravenous catheter (Becton-Dickinson, Sandy, Utah, USA). The inspired isoflurane concentration was decreased to 1.5%, and the lungs were mechanically ventilated at a

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rate of 50 breaths/min with a delivered tidal volume of 3 ml. Temperature was monitored by rectal probe and held constant at 37 ± 0.2 °C with a surface heating/cooling system.

ICH Model

The mouse ICH model [45, 49] was adapted for rats. After anesthesia induction and tracheal intubation, the animal's head was secured in a stereotactic frame. The scalp was incised, and a burr hole was created at 3 mm left lateral at bregma. A 5.0-μl syringe (Hamilton, Reno, Nev., USA) with a 26-gauge needle was mounted on a micromanipulator. The bevel of the needle was advanced to a depth of 6 mm from the cortical surface. Type IV-S clostridial collagenase (Sigma, St. Louis, Mo., USA; 0.5 U in 2.5 μl 0.9% normal saline) was injected over 6 min, and the needle was held in place motionless for an additional 10 min. After slowly withdrawing the needle, 0.25% bupivacaine was instilled in the wound, the incision was closed with suture, anesthesia was discontinued, and animals were allowed to recover spontaneous ventilation with subsequent tracheal extubation. Following recovery in a warm, nonstimulating environment, rats were returned to their cages, and allowed free access to food and water.

Preparation and Administration of Progesterone

Rats were given 0.3 ml of progesterone or vehicle intraperitoneally at 2 h after injection and subcutaneously on the nape of the neck at 5, 24, 48, and 72 h after injury. Progesterone (8 mg/kg; P0130, Sigma-Aldrich, Saint Louis, Mo., USA) was dissolved in 8 mg/ml sesame oil (S3547, Sigma-Aldrich). Vehicle treatment was volume-equivalent sesame oil only. Administration of progesterone earlier than 2 h after ICH onset is not clinically feasible; therefore, this time point was chosen for initial dosing.

Neurobehavioral Testing

An automated rotarod (Ugo Basile, Varese, Italy) was used to assess vestibulomotor function. Before hemorrhage induction, rats underwent 3 consecutive days of conditioning, with 3 trials of accelerating rotational speed each day. Baseline latency was recorded as the average time to fall from the rotating cylinder on the day before injury. After injury, rats underwent rotarod testing on days 1, 3, 5, and 7, again with 3 trials of accelerating rotational speed each day with intertrial intervals of 15 min. Average latency to fall from the rod was computed for each day [50].

The neuroseverity score was recorded on days 1, 3, 5, and 7 after injury, as previously described [51]. Day 1 was chosen as the first time point because hemorrhage volume is known to reach steady state by this time [52]. The 0–48 scoring scale includes general status, simple motor, complex motor, and sensory components. The general status score (0–12) was based on spontaneous activity, body symmetry, and gait. Front limb symmetry, circling movements, circling while holding tail, and hind limb placement were used to score simple motor function (0–14). The complex motor function score (0–8) was based on vertical screen climbing and beam walking. The sensory score (0–14) was derived from fore/hind limb, vibrissae, trunk, and face touch. Sensory tests examined function from both cerebral hemispheres.

Rats underwent corner turn testing on days 1, 3, 5, and 7 after injury. Rats were placed in a corner with a 30-degree angle, and could turn left or right spontaneously. The turn direction was recorded. Only full turns along the walls were recorded, i.e. horizontal turns and ventral tucks were excluded. Corner turn testing was repeated 10 times per day with 30-second intervals between each trial. The percentage of right turns was used for analyses. To avoid development of aversion to the natural turning response, rats were briefly allowed free range after each trial [53].

Measurement of Brain Water Content

Rats were anesthetized and decapitated. Brains were harvested and divided along the interhemispheric fissure, and the hindbrain was discarded. Each hemisphere was weighed immediately (wet weight). Hemispheres were dehydrated over 24 h at 105 °C, and then re-weighed (dry weight). Brain water content was calculated as wet weight – dry weight/wet weight × 100 [54].

Histological Measurement of Hematoma Volume

After anesthesia induction, rats were decapitated, and brains were removed, flash frozen in 2-methyl butane (–20 °C), and stored at –80 °C. Coronal sections 20 μm thick were serially taken at 800-μm intervals over the rostral-caudal extent of the lesion. The sections were stained with hematoxylin and eosin (HE), and the lesion area was measured by digitally sampling stained sections with an image analyzer (MCID Elite™, Interfocus Imaging, Linton, UK). Hematoma volumes (mm^3) were computed as running sums of the lesion area multiplied by the known interval between sections (800 μm) over the extent of the lesion, and expressed as an orthogonal projection [54].

Immunohistochemistry

Anti-ionized calcium-binding adapter molecule 1 (Iba-1) is a microglia and macrophage marker. Iba-1 polyclonal antibody was used for immunohistochemical staining on day 7 after ICH [55–57]. After anesthetic induction, rats were subjected to transcardial perfusion with 100 ml phosphate-buffered saline (PBS), and switched to 150 ml 4% formaldehyde. Brains were removed and immersion-fixed in 4% formaldehyde at 4 °C for 24 h, and then transferred to 30% sucrose/1× PBS, and stored at 4 °C for 48–72 h. Frozen coronal brain sections (40 μm) were collected on a freezing sliding microtome. Floating brain sections were incubated in 1% hydrogen peroxide, permeabilized by 0.1% saponin, and blocked with 10% goat serum. Sections were incubated overnight with anti-Iba-1 antibody specific for microglia and macrophage (1:10,000; Wako Chemicals, USA). Biotinylated goat anti-rabbit immunoglobulin G secondary antibody (1:3,000; Vector Laboratories Inc., Burlingame, Calif., USA) was then applied for 2 h, followed by treatment with avidin-biotin-peroxidase complex for 1 h (ABC kit; Vector Laboratories). Staining was visualized with diaminobenzidine (DAB kit; Vector Laboratories). After mounting onto slides, all sections were counterstained with hematoxylin (Fisher Scientific, Fair Lawn, N.J., USA).

Cell Quantification and Image Analysis

Stereological analysis was performed on a Nikon 218912 light microscope interfaced with the StereoInvestigator software package (MicroBrightField; Williston, Vt., USA). The number of Iba-1-positive cells per volume of perihematomal areas was estimated using the optical fractional method [58]. The perihematomal area was defined as a 375-μm band around the hematomal circumference. Before counting, all slides were coded to avoid experimenter bias. Six sagittal sections (40 μm), spaced 8 sections apart, were chosen for cell counting. For microglial quantification, the sampling grid was 399 (X) × 368 (Y) μm, and cells were counted with-

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in a probe volume, defined by the counting frame (80 × 80 μm), at ×20 magnification. The total number of immunopositive cells was calculated per hippocampal volume of 1,920-μm thickness.

Cytokine Concentration by Enzyme-Linked Immunosorbent Assay

After anesthetic induction, rats were perfused transcardially with 100 ml PBS. Brains were removed and divided along the interhemispheric fissure, flash frozen in liquid nitrogen, and stored at –80 °C. Pulverized injured hemispheres were sonicated on ice for 20 s in homogenization buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100) and complete protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Brain homogenates were incubated on ice for 20 min, and then spun at 10,000 *g* at 4 °C for 10 min. The supernatant was removed, aliquoted, and stored at –80 °C for protein analysis. Total protein concentrations were measured using a bicinchoninic acid protein assay reagent kit (Pierce Biotechnology, Rockford, Ill., USA). Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) concentrations in brain homogenates were determined using a rat IL-6 enzyme-linked immunosorbent assay (ELISA) kit (Cat. No.: ELR-IL6-CL) and rat TNF-α ELISA kit (Cat. No.: ELR-TNFα-CL), respectively, according to the manufacturer's instructions (RayBiotech Inc., Norcross, Ga., USA).

Western Blot Analysis

Brain homogenate samples, loaded equally and resolved on 4–20% SDS polyacrylamide gels, were transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBS with 0.1% Tween-20 and 5% dry milk for 1 h at room temperature, and then incubated at 4 °C overnight with primary antibodies against phosphor-nuclear factor-κB (NF-κB) p65, GAPDH (Cell Signaling Technology Inc. Beverly, Mass., USA), and toll-like receptor-4 (TLR-4; Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA). After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) at 1/10,000 dilution for 1 h at room temperature, the signal was detected using Western Dura Extended Duration Substrate (Thermo Scientific, Rockford, Ill., USA), and imaged with an HD2 CCD camera (Alpha Innotech, San Leandro, Calif., USA). When multiple probing was needed, membranes were stripped off the immunoglobulin using restore stripping buffer (Thermo Scientific) before re-probing.

Magnetic Resonance Imaging

All MRI experiments were carried out on a 7-tesla preclinical Bruker (Billerica, Mass., USA) Biospec 70/30 USR MRI scanner with Paravision 5.1 interface and quadrature transmit-receive volume coil (72 mm). Animals were placed prone on the holder and transitioned inside the magnet with the animal head positioned at the magnet isocenter. Anesthesia was induced with 5% isoflurane and maintained at 1.5% isoflurane in 40% O_2 and 60% N_2 via nose cone. Cardiac and respiratory rates were monitored via a pressure transducer and temperature using an MRI-compatible rectal thermistor. Body temperature was maintained at 37 °C using a continuous feedback circulating hot water reservoir. MRI for each animal was performed using the following protocol: (1) field map shimming for optimization of B_0 field homogeneity; (2) fast spin echo-based T_2-weighted acquisitions with echo time (TE)/repetition time (TR) = 12/4,200 ms, field of view (FOV) = 3 × 3 cm, matrix 128 × 128, 32 slices, and 1-mm slice thickness for anatomy; (3) echo planar-based acquisitions for diffusion and perfusion-weighted mapping with TE/TR = 38/8,000 ms, FOV 3 × 3 cm, matrix 80 × 80, 32 slices, 1-mm thickness, 1 segment, 4 averages, and B values of 0, 50, 100, 200, and 1,000 s/mm^2; (4) fast low-angle shot gradient echo-based 3-dimensional isotropic T1-weighted acquisitions with TE/TR = 10/30 ms, flip angle of 20°, FOV = 4 × 4 × 4 cm, and matrix 128 × 128 × 128 for volumetric calculations. All data sets were configured to Digital Imaging and Communications in Medicine format and postprocessed offline using Osirix software (http://www.osirix-viewer.com) and Osirix integrated analytical algorithms (Pixmeo Sarl, Bernex, Switzerland). Hemorrhage volumes were calculated from regions of interest manually obtained on individual two-dimensional slices extracted from isotropic 3-dimensional fast low-angle shot data sets. Apparent diffusion coefficient maps were calculated from the echo planar acquisitions, with B values of 100 and 200 s/mm^2 used for perfusion weighting and B values of 0 and 1,000 s/mm^2 for diffusion weighting. Regions of interest for diffusion and perfusion measurements were calculated for the whole brain excluding the region of hemorrhage.

Acute Physiology and Blood Pressure Measurement

After anesthesia induction and tracheal intubation, the tail artery was cannulated to monitor arterial blood pressure and collect arterial blood samples. A pressure transducer was connected to the artery catheter, and blood pressure was monitored continuously and recorded at 15 min before ICH induction and at 15-min intervals thereafter until 3 h after injury [59]. Blood samples were taken from the artery catheter for blood gas and glucose concentrations 15 min before and at 1.5 and 3 h after ICH injury.

Laser Doppler Flowmetry Measurement

During physiological monitoring, cerebral blood flow was monitored by laser Doppler flowmetry (LDF; PeriFlux 4000 System, Probe 407; Perimed Instruments, Järfälla, Sweden) for the hemisphere contralateral to ICH. A small burr hole was drilled 1 mm posterior to bregma and 0.5 mm lateral to midline, and the micro-Doppler probe was positioned above the dura mater in a holder glued to the bone. LDF was monitored continuously and recorded at 15 min before ICH induction and at 15-min intervals thereafter until 3 h after injury [60, 61].

Statistical Analysis

Animal numbers were based on power to detect a difference in rotarod latencies between progesterone- and vehicle-treated rats on days 1–7 after injury, with β = 0.8. Independent sample size calculations indicated that 10–12 animals per group would be required to detect a 50% difference between treatment and vehicle in 2 groups (male or female). The number of animals for other experimental groups was based on experience and minimized to an acceptable degree. Repeated-measures analysis of variance (RM-ANOVA) with time as the repeated variable was used to compare rotarod performance and neuroseverity score with test group effect as a function of time. Bonferroni correction was used for the repeated measures technique in RM-ANOVA, and Student's t test to compare hematoma volume, brain water content, immunohistochemistry, cytokine mRNA concentration, cerebral perfusion/diffusion, ELISA, and Western blot quantification. One-way RM-ANOVA was used to compare MRI hemorrhage volume.

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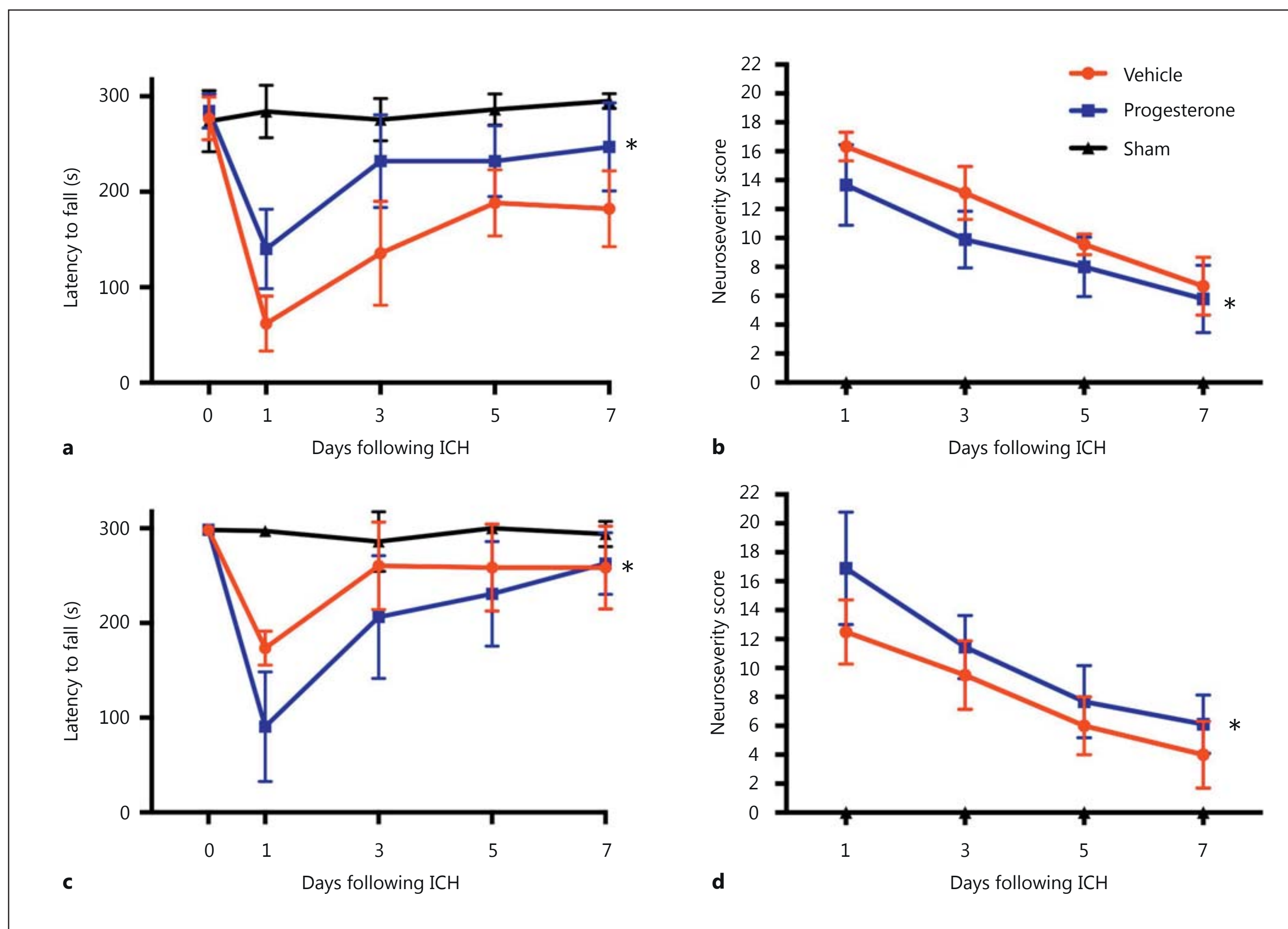

Fig. 1. Progesterone improves neurobehavioral outcomes after ICH in male rats. Outcome after ICH was improved in male rats after daily progesterone treatment (8 mg/kg) for 3 days, as assessed by rotarod latency (p = 0.001; n = 10/group; **a**) and neuroseverity score (p = 0.0097; n = 10/group; **b**) compared to volume-equivalent vehicle treatment. In female rats, outcomes were worse after progesterone treatment, as assessed by rotarod latency (p = 0.028; n = 10/group; **c**) and neuroseverity score (p = 0.008; n = 10/group; **d**). Sham-treated animals performed the same as uninjured animals in both rotarod latency and neuroseverity tests. Values are expressed as mean ± SD. * p < 0.05 vs. vehicle-treated group. A neuroseverity score of 0 = no deficit.

Results

Neurobehavioral Recovery after ICH and Progesterone Treatment

To assess efficacy as a potential neurotherapeutic agent for acute ICH, progesterone (8 mg/kg) was administered in repeated doses after injury. Progesterone-treated male rats showed improved neurobehavioral outcome, as measured by rotarod latency (p = 0.001) and neuroseverity score (p = 0.0097), throughout the study period compared to vehicle (fig. 1a, b). However, progesterone-treated females demonstrated shorter rotarod latencies compared to vehicle-treated females until day 7 after ICH (fig. 1c; p = 0.028). Further, neuroseverity scores were worse in progesterone-treated females compared to vehicle (fig. 1d; p = 0.008). In both male and female rats, no significant differences between progesterone- and vehicle-treated animals were detected in the corner turn test over the first 7 days after ICH (data not shown).

Brain Water Content and Hematoma Volume after ICH and Progesterone Treatment

Cerebral edema formation is associated with neurobehavioral outcomes. The degree of cerebral edema forma-

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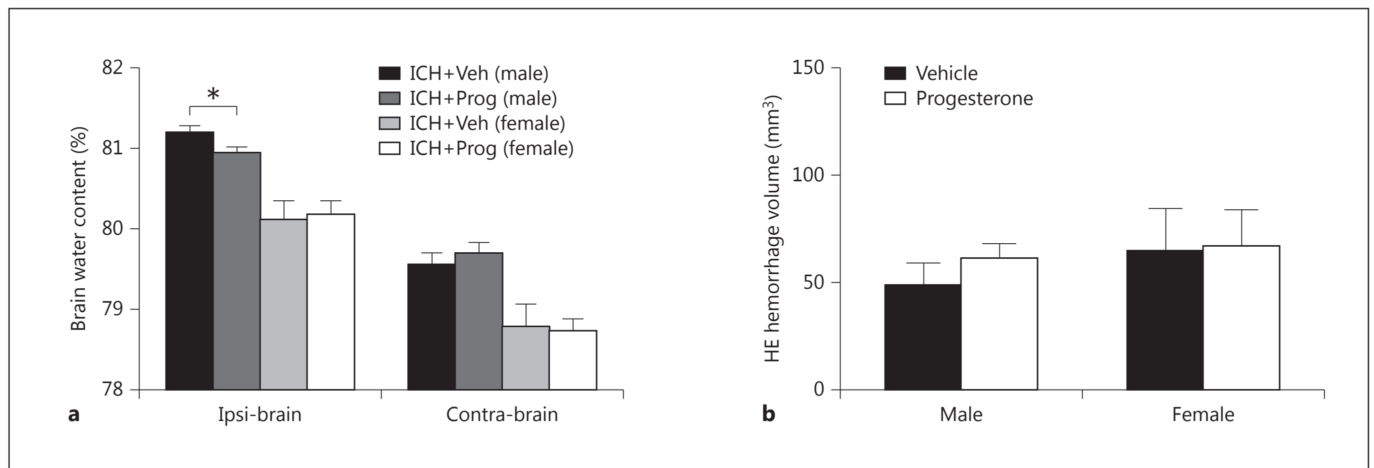

Fig. 2. Progesterone reduces cerebral edema but does not affect hematoma volume formation at 24 h after ICH in male rats. **a** Male rats treated with daily progesterone (8 mg/kg) for 3 days after ICH had less brain water in the ipsilateral (80.9 ± 0.2 vs. 81.3 ± 0.1%; p = 0.044; n = 8/group), but not the contralateral (79.56 ± 0.14 vs. 79.70 ± 0.13%; p = 0.485; n = 8/group), hemisphere compared to vehicle-treated animals. Female ICH rats showed no effect after progesterone treatment in the ipsilateral hemisphere (80.11 ± 0.24 vs. 80.18 ± 0.13 mm^3; p = 0.829; n = 5/group) or the contralateral hemisphere (78.79 ± 0.28 vs. 78.73 ± 0.15%; p = 0.841; n = 5/group) compared to vehicle. **b** Hematoma volume, as measured by HE staining, was not changed after progesterone treatment compared to vehicle in male (61.3 ± 2.8 vs. 49.1 ± 4.5 mm^3; p = 0.052; n = 5/group) or female (67.3 ± 7.4 vs. 65.1 ± 8.7 mm^3; p = 0.853; n = 5/group) rats. Values are expressed as mean ± SD. * p < 0.05.

tion after ICH was assessed in progesterone- and vehicle-treated male and female rats (fig. 2a). Male rats treated with progesterone had lower brain water content in the ipsilateral (p = 0.044), but not the contralateral (p = 0.485), hemispheres compared to the vehicle-treated group. In female rats, no difference in brain water content in either the ipsilateral or contralateral hemisphere was observed between treatment groups.

Hematoma volume is a key mediator of outcome after human ICH [62]. HE staining of brains at 24 h after ICH showed no difference in hematoma volume between progesterone- and vehicle-treated male or female rats (fig. 2b).

Microglial Activation/Macrophage Recruitment after ICH and Progesterone Treatment

Microglial activation and macrophage recruitment are known mediators of cerebral edema formation and neurobehavioral outcome after ICH [63, 64]. Iba-1 staining was performed at 7 days after ICH induction in the male rats. The number of Iba-1-positive cells in the ipsilateral perihematomal area decreased in the male rats after progesterone treatment compared to vehicle (fig. 3; p < 0.001).

Proinflammatory Transcription Factors and Cytokine Levels after ICH and Progesterone Treatment

Proinflammatory cytokines IL-6 and TNF-α promote neuroinflammation via NF-κB after ICH [54, 64, 65]. At 24 h after injury, phosphor-NF-κB p65 expression was downregulated in male (p = 0.0038) but not female rats, while TLR-4 expressions remained unchanged (Western blot; fig. 4a–c). IL-6 and TNF-α were not altered in male or female rats treated with progesterone (ELISA; fig. 4d, e).

Cerebral Perfusion and Diffusion after ICH and Progesterone Treatment as Measured by MRI

Chronic hypertension is associated with ICH incidence and outcome [66, 67]. To examine changes after progesterone treatment in the setting of comorbid hypertension, MRI was performed in male SHRs to corroborate findings in nonhypertensive rats used in the other experiments. Consistent with HE findings, MRI hematoma volume in progesterone-treated SHRs was not different when compared to vehicle-treated SHRs and control WKRs at 4 and 24 h after ICH (fig. 5a, b). At 4 h after ICH, progesterone-treated SHRs showed increased cerebral perfusion (p = 0.0427) but not diffusion compared to vehicle-treated SHRs and control WKRs. Differences in perfusion and diffusion were not seen at 24 h (fig. 5c, d).

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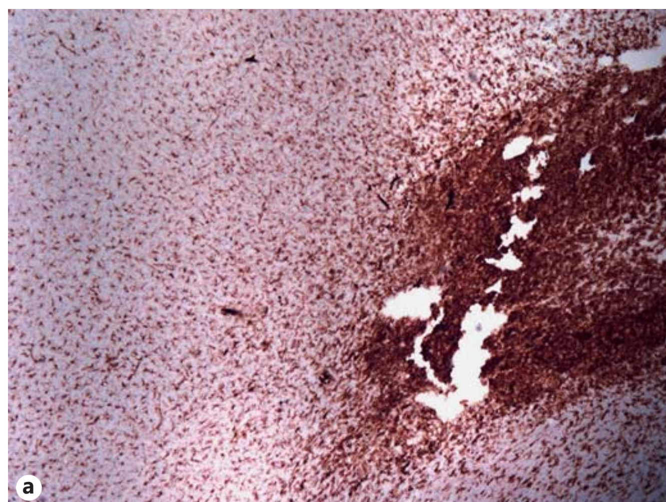

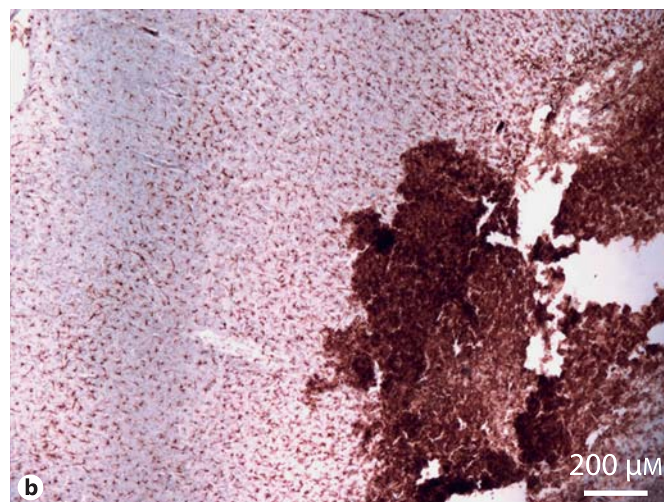

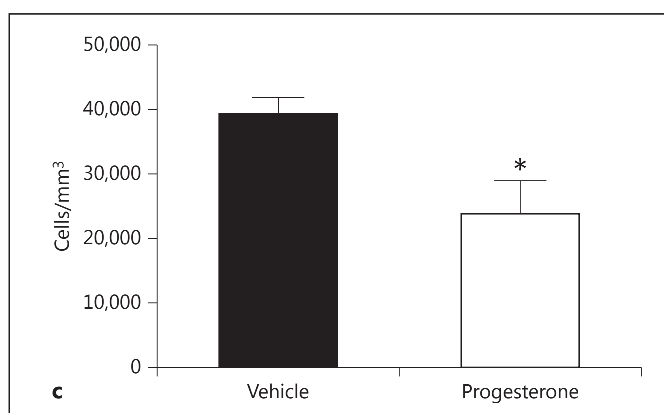

Fig. 3. Progesterone reduces microglial activation/macrophage recruitment after ICH in male rats. At 7 days after ICH, Iba-1 staining for microglial activation and macrophage recruitment was greater in vehicle-treated (39,486 ± 2,360 cells/mm^3; **a**) than progesterone-treated (8 mg/kg daily for 3 days; 23,956 ± 4,999; **b**) male rats after ICH ($p < 0.001$; n = 5/group). **c** Values are expressed as mean ± SD. * $p < 0.001$.

Acute Physiology after ICH and Progesterone Treatment

To mimic typical ICH clinical presentation, physiological measurements between progesterone- and vehicle-treated SHRs and WKRs (to control for hypertension) were compared. All SHRs, regardless of treatment group, had a significantly higher systolic blood pressure through the first 3 h after injury compared with WKRs. Progesterone did not alter systemic blood pressure in SHRs. Neither LDF nor arterial blood gas concentrations were different between groups (table 1).

Discussion

Few studies have investigated the effects of exogenous progesterone after ICH; however, neuroprotective and anti-inflammatory effects of progesterone following TBI, ischemia, and SAH are well established. In preclinical TBI, progesterone treatment improved motor performance, decreased brain edema, diminished free radical formation and lipid peroxidation, and downregulated proapoptotic and upregulated antiapoptotic enzymes in both male and female injury models [10–22, 68–72]. Similarly, progesterone promoted functional recovery in ischemic brain injury models [31–33, 73, 74] by reducing proinflammatory cytokine formation, decreasing infarct volume, minimizing hemorrhagic transformation, and maintaining blood-brain barrier integrity [23–30]. In SAH, progesterone attenuated vasospasm, reduced lipid peroxidation, and prevented apoptosis [35–38]. While mechanically different, ICH shares some similarities with trauma, ischemia, and SAH in pathophysiology, particularly in secondary neuroinflammation. Therefore, we tested the hypothesis that progesterone improves recovery after ICH, and found that progesterone given 2 h after ICH injury onset improved recovery in male, but not female rats.

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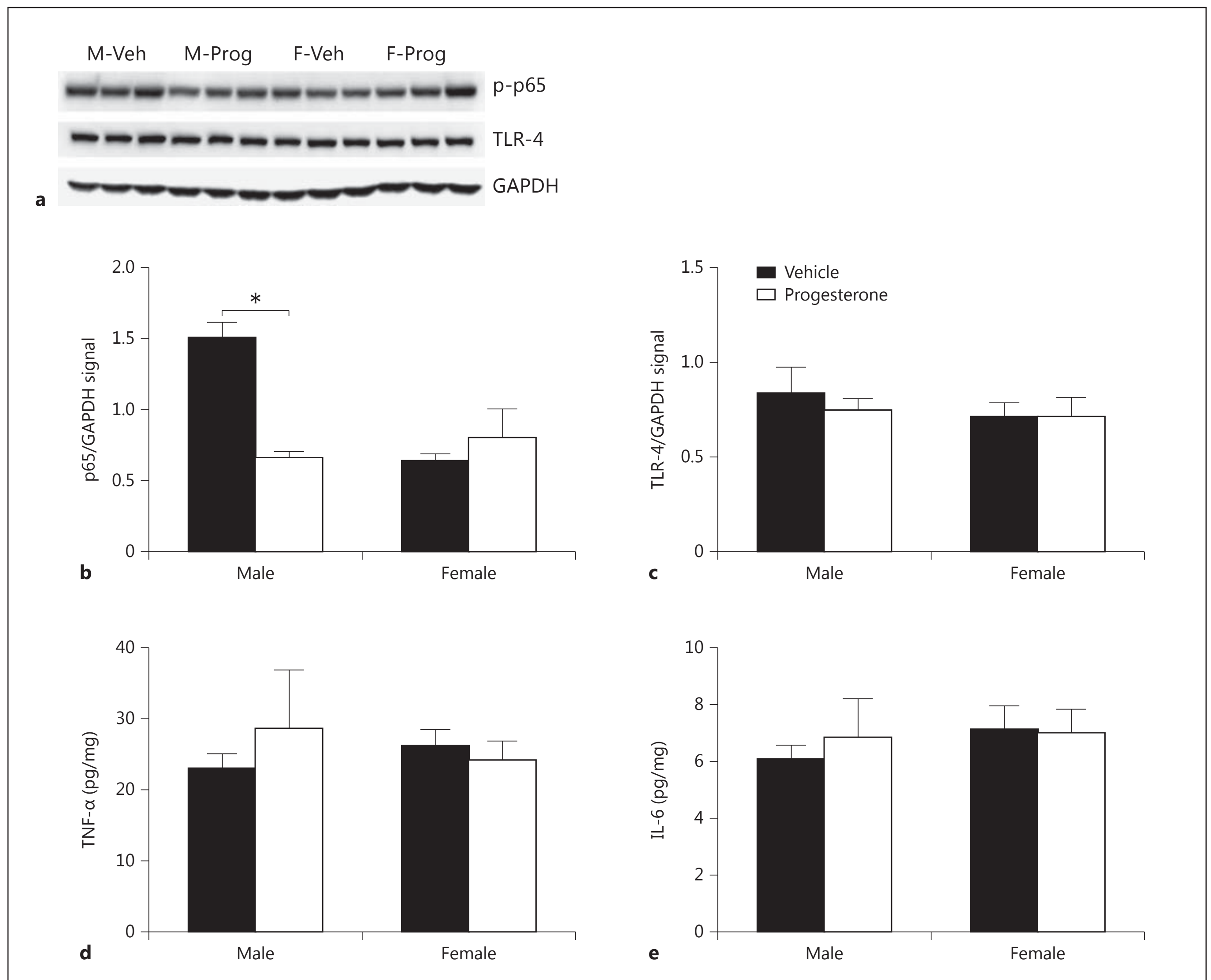

Fig. 4. Proinflammatory transcription factor and cytokines after ICH. **a**, **b** At 24 h after ICH, proinflammatory transcription factor phosphor-NF-κB (p-p65) expression measured by Western blot (n = 3/group) was downregulated by progesterone treatment (8 mg/kg daily for 3 days) in male [1.05 ± 0.060 vs. 0.666 ± 0.023 p-p65/glyceraldehyde 3-phosphate dehydrogenase (GAPDH); p = 0.0038], but not female (0.641 ± 0.027 vs. 0.802 ± 0.12 p-p65/GAPDH; p = 0.2476) rats, compared to vehicle treatment. TLR-4 (**a**, **c**) measured by Western blot (n = 3/group) and proinflammatory cytokines TNF-α (**d**) and IL-6 (**e**) measured by ELISA (n = 3/group) were not significantly reduced after progesterone treatment (8 mg/kg daily for 3 days) compared to vehicle in male or female rats at 24 h after ICH (TLR-4: male, 0.837 ± 0.79 vs. 0.748 ± 0.033 TLR-4/GAPDH, p = 0.3599, and female, 0.716 ± 0.040 vs. 0.714 ± 0.058 TLR-4/GAPDH, p = 0.9773; IL-6: male, 6.87 ± 0.78 vs. 6.10 ± 0.28 pg/mg, p = 0.4083, and female, 7.02 ± 0.48 vs. 7.15 ± 0.47 pg/mg, p = 0.859; TNF-α: male, 23.07 ± 1.15 vs. 28.66 ± 4.78 pg/mg, p = 0.319, and female, 24.3 ± 1.50 vs. 26.3 ± 1.24 pg/mg, p = 0.3485). Values are expressed as mean ± SD. * p < 0.05.

Rats were weight matched across sex in order to normalize to brain weights, an important indicator of CNS maturity. To control for high endogenous progesterone concentration across the female rats, injury was performed at the diestrous stage [47, 48]. Improved rotarod performance and neuroseverity score persisted through day 7 after ICH. However, no difference in corner turn testing was seen in males or females as a function of treatment regimen. This seeming contradiction is likely due to the high sensitivity of the test to sensorimotor deficit,

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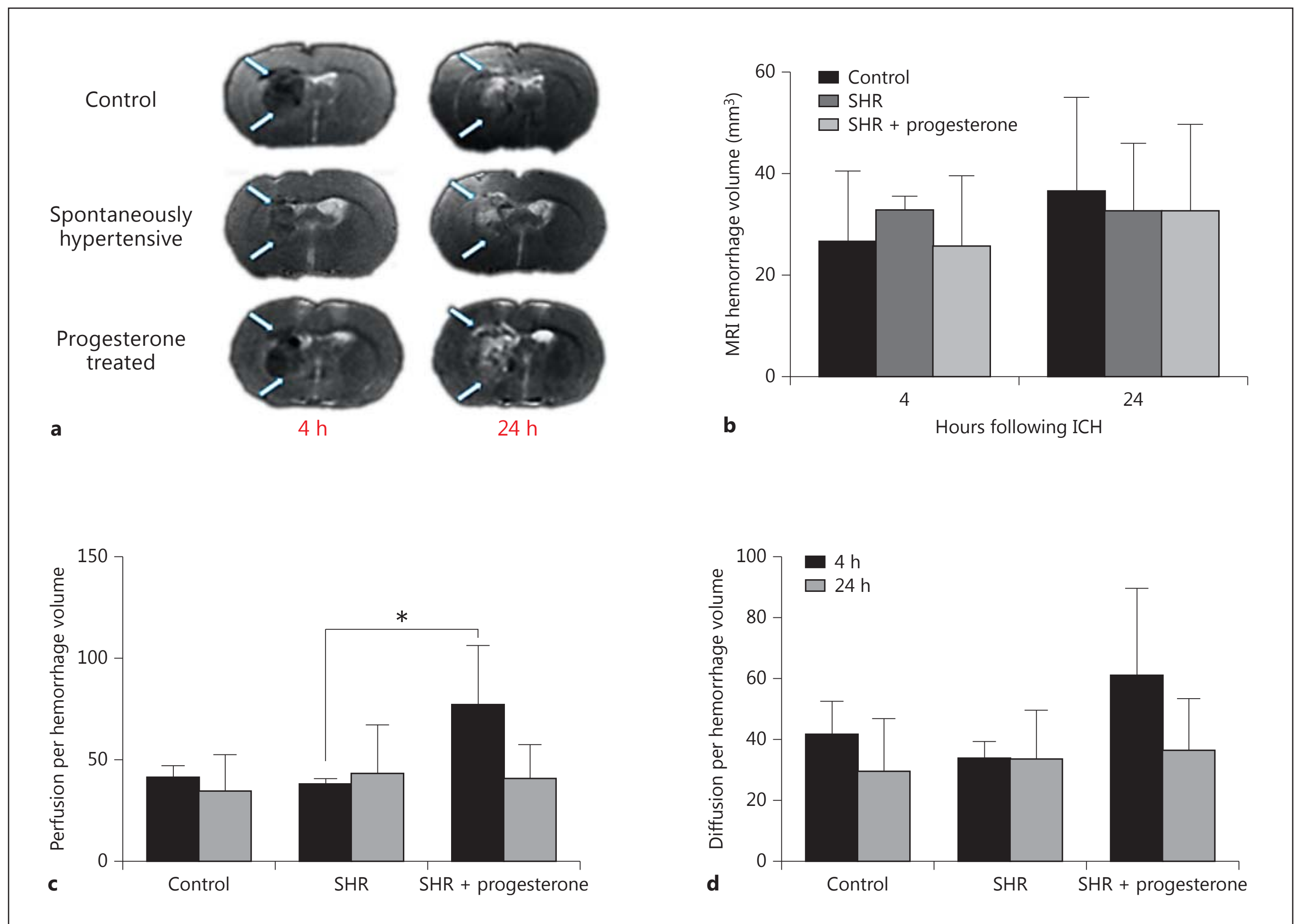

Fig. 5. Progesterone affects MRI biomarkers at 4 and 24 h after ICH in male rats. **a** Sample T2-weighted MRI (n = 3/group) at 4 and 24 h after ICH in control (WKR), vehicle-treated SHRs, and progesterone-treated (8 mg/kg at 2 and 5 h after ICH) SHRs. Hypointense 'dark' blood products at 4 h are consistent with deoxyhemoglobin and/or intracellular methemoglobin, while their conversion to hyperintense 'bright' blood products likely represents extracellular methemoglobin. **b** Consistent with histological findings, hemorrhage volumes by MRI were not different between progesterone-treated (SHR + progesterone) SHRs, vehicle-treated SHRs (SHR + vehicle), and male WKRs (control) at 4 h (25.8 ± 13.8 vs. 32.9 ± 2.55 vs. 26.7 ± 13.9; p = 0.7114) and 24 h (32.7 ± 17.0 vs. 32.7 ± 13.3 vs. 36.7 ± 18.4; p = 0.9549) after ICH. Progesterone-treated SHRs had increased cerebral perfusion [76.5 ± 29.4 vs. 37.8 ± 2.85 vs. 41.2 ± 5.88; p = 0.0427 (progesterone- vs. vehicle-treated SHR); **c**] but not diffusion (61.1 ± 28.6 vs. 33.8 ± 5.58 vs. 41.6 ± 11.2; p = 0.0892; **d**) at 4 h after ICH. Differences in perfusion and diffusion were no longer seen at 24 h. Values are expressed as mean ± SD. * $p < 0.05$.

which hinders any observation of more subtle recovery within the first 7 days; rats continue to exhibit side preference for up to 2 weeks after ICH [53].

Although hematoma volume or evolution was not affected by progesterone treatment, neurobehavioral improvement was associated with reduced brain water content at day 1 after injury. Cerebral diffusion by MRI (vasogenic cerebral edema) was not changed at 4 and 24 h after injury comparing vehicle- to progesterone-treated male rats. Progesterone-induced reduction in active water diffusion may not be detected by MRI after ICH, but it reduces brain water accumulation measured at 24 h, and this effect may persist beyond this time point.

Cerebral edema after ICH is believed to be an extension of neuroinflammation. Major contributors to neuroinflammation include activation of immunomodulatory microglia and their subsequent release of cytokines [75–77]. In the current study, progesterone decreased microglial and macrophage activation. However, Iba-1 staining does not distinguish between microglia and macrophages.

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Table 1. Acute physiology after ICH injury in male rats

	WKR (n = 3)	SHR (n = 3)	SHR + Prog (n = 3)
Arterial pH			
Before ICH	7.38±0.01	7.38±0.01	7.37±0.01
1.5 h	7.36±0.01	7.37±0.01	7.36±0.01
3 h	7.35±0.01	7.37±0.01	7.35±0.01
$PaCO_2$, mm Hg			
Before ICH	41.3±3.0	38.4±1.9	36.2±2.4
1.5 h	44.4±6.2	37.2±1.9	36.0±1.4
3 h	46.0±4.2	38.1±0.8	37.6±2.5
PaO_2, mm Hg			
Before ICH	94.2±7.6	103.0±4.6	104.7±2.1
1.5 h	110.5±4.9	105.0±3.6	112.0±4.4
3 h	119.0±1.4	105.3±2.5	110.0±5.0
Glucose, mg/dl			
Before ICH	136±2	139±7	139.7±6
1.5 h	137±3	144±2	146.7±9
3 h	136±9	142±6	153.7±7
Systolic blood pressure, mm Hg			
Before ICH	93±4	129±6	128±14
1.5 h	69±16	118±7	108±5
3 h	62±10	109±5	111±4
Cerebral blood flow (laser Doppler flow)			
Before ICH	68±4	66±2	73±4
1.5 h	138±2	131±2	134±4
3 h	130±10	134±7	132±4

Measurements were taken before ICH, and at 1.5 and 3 h after injury (n = 3).

Regardless, these data are consistent with previous reports that progesterone exerts immunomodulatory effects on microglia and macrophages [14, 70, 78–80]. Consistent with previous reports, progesterone was found to decrease transcription factor p-NF-κB p65 expression [54, 64, 65]. This decrease was not accompanied by a decrease in the proinflammatory cytokines IL-6, TNF-α, or TLR-4 at the same 24-hour time point. Thus, decreased expression of downstream products are likely evident at later time points. Further investigation of these complicated interactions is warranted.

Additionally, progesterone increased cerebral vessel perfusion acutely at 4 h with return to baseline by 24 h. Increased perfusion was not accompanied by a change in systemic blood pressure or contralateral hemispheric LDF. Progesterone is known to produce vasodilation in superficial pial vessels after ischemic brain injury, which was postulated to decrease infarction in the striatum [81–83]. The mechanisms by which progesterone affects cerebral perfusion after ICH, however, remain unclear.

The current study was the first to test progesterone treatment after ICH in female rats. Compared to the beneficial effects seen in males, progesterone paradoxically worsened neurobehavioral outcome in gonad-intact females. This could be explained by the combination of exogenous and endogenous hormones. Progesterone at higher concentrations causes increased modulation of γ-aminobutyric acid type A ($GABA_A$) and other receptors leading to sedation [70, 84, 85]. In addition, co-administration of progesterone and estrogen decreases the neuroprotective effect of estrogen [86, 87]. Another possibility is that the female rats were experiencing progesterone withdrawal and tapering of the doses could reduce this effect [88]. Other complex interactions between female gonadal hormones may also explain the observed deficit in neurobehavioral recovery [89, 90].

Recent reports point out that older women have increased incidence and worse severity of ICH and ischemic stroke as compared to younger women and age-matched men [9, 91]. Our data here show that endogenous progesterone in the young female rats may provide a sufficient neuroprotective effect alone. Exogenous administration paradoxically worsens outcome, supporting the hypothesis that an ideal brain concentration of progesterone should be sought for neuroprotection. This further highlights the possibility that progesterone given to postmenopausal female rats, either through age or ovariectomy, might produce similar beneficial effects as seen in the male cohort. The effect of different progesterone concentrations in aged and ovarectomized female animals is an important follow-up to the current study.

Our finding that progesterone was not efficacious in females may be important for clinical trial design. Contrary to various preclinical studies demonstrating efficacy of progesterone in TBI, the data from the ProTECT III trial did not find efficacy in humans [15, 92–94]. A single study in rats does not fully preclude potential progesterone efficacy in human females. However, failure to account for sex-specific effects in clinical trials may dilute any efficacy found in the overall study population. Indeed, males and females could require different doses for maximum effectiveness, and optimal progesterone doses may also be different for pre- and postmenopausal women. Future dose-response studies can further clarify this question and potentially resolve the observed discrepancies in progesterone's neuroprotective efficacy between preclinical studies and clinical trials.

The 8 mg/kg dose was selected based on previous reports of efficacy in ICH. While the present results are encouraging, optimal dosing strategy for maximal benefit in

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male ICH rats, and the corresponding in vivo CNS concentration must be established before human translation. While unaddressed here, progesterone's ICH-specific mechanism(s) of action should be further defined. It would be of high interest to use specific receptor blockers, including classical nuclear receptors, membrane progesterone receptors, membrane progesterone receptor component 1, and $GABA_A$ to assess this [22]. Finally, progesterone's efficacy in aged animals and in other species should be examined prior to any attempts at translation.

Conclusion

The current findings demonstrate that progesterone decreases early cerebral edema and neuroinflammation while improving neurobehavioral recovery after ICH and, thus, is a promising therapeutic agent for translation into human trials. However, exogenous progesterone could be detrimental in reproductive females, though the mechanism for this remains unclear.

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Disclosure Statement

The authors declare no conflicts of interest.

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