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ORIGINAL ARTICLE

Delayed progesterone treatment reduces brain infarction and improves functional outcomes after ischemic stroke: a time-window study in middle-aged rats

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We evaluated the neuroprotective effects of delayed progesterone (PROG) treatment against ischemic stroke-induced neuronal death, inflammation, and functional deficits. We induced transient focal cerebral ischemia in male rats and administered PROG (8 mg/kg) or vehicle intraperitoneally at 3, 6, or 24 hours post occlusion, subcutaneously 5 hours later and then every 24 hours for 7 days. Behavioral outcomes were evaluated over 22 days. Infarct size and other biomarkers of injury were evaluated by cresyl violet staining, and matrix metalloproteinase-9 (MMP-9), glial fibrillary acidic protein (GFAP), and vascular endothelial growth factor (VEGF) by immunofluorescence. Progesterone treatment started at 3 and 6 hours post occlusion significantly ($P < 0.05$) improved behavioral performance at all time points (74.01%) and reduced infarction volume (61.68%) compared with vehicle. No significant difference was observed between the 3 and 6 hour PROG treatment groups. Matrix metalloproteinase-9 and VEGF were upregulated in the PROG groups compared with vehicle. Glial fibrillary acidic protein expression was increased in the vehicle group but markedly lower in the PROG groups. Treatment delayed for 24 hours did not significantly improve functional outcomes or reduce infarction volume. We conclude that, under the right treatment conditions, PROG treatment delayed up to 6 hours can improve functional deficits and reduce brain infarction, possibly by modulating GFAP, VEGF, and MMP-9 expression.

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INTRODUCTION

Transient ischemia is the most common form of stroke and accounts for 87% of all strokes. To date, no clinical trial has led to a successful pharmacological intervention for this disease. Tissue plasminogen activator is the only FDA-approved drug to treat stroke patients, but it can be used in at most 5% of the stroke population. This is in part because of its limited 3- to 4.5-hour time window for treatment, after which its efficacy falls rapidly,[1] and in part to its increased risk of blood thinning and bleeding leading to intracranial hemorrhage. Further, tissue plasminogen activator has no efficacy in hypertensive subjects, so its use is likely to be limited in the substantial proportion of patients with a history of hypertension.[2] There is still no clinically safe and effective neuroprotective treatment that can be given to the remaining 95% of stroke patients.

The Stroke Therapy Academic Industry Roundtable has issued recommendations for clinically relevant preclinical testing for stroke treatments.[3] The Stroke Therapy Academic Industry Roundtable recommendations include determining dose–response relationships, extending therapeutic time windows, and testing efficacy in multiple stroke models including permanent and transient ischemia, aged animals, and animals with comorbidities. They also recommend improvements in defining various behavioral and histologic outcomes. We sought to address a number of these parameters in the present study.

There is now a very substantial literature showing that the neurosteroid hormone progesterone (PROG) is safe and neuroprotective. It has already been shown in two independent phase II clinical trials for mild–to-severe traumatic brain injury (TBI)[4,5] to significantly reduce mortality and improve functional outcomes. Two phase III multicenter clinical trials to treat brain trauma patients with PROG are nearing completion (Protect III, 2011, http://www.clinicaltrials.gov/NCT00822900; SyNAPSe, 2011, http://www.synapse-trial.com) and both are continuing beyond their interim analyses with no safety concerns having been reported.

Earlier studies from our laboratory and others have shown promising neuroprotective effects of PROG against TBI and stroke in several animal models. Progesterone treatment produced a decrease in infarct volume, inflammatory cytokine expression, apoptosis, and cerebral edema leading to improved functional outcomes after stroke and TBI.[6–12] Recently, we found that PROG treatment may be beneficial not only in the treatment of ischemic stroke itself but also in fighting poststroke infection, a major comorbidity after stroke.[13] In another recent study, we showed that after stroke, a combination of PROG with vitamin D hormone afforded more neuroprotection than PROG treatment alone.[14]

Addressing the Stroke Therapy Academic Industry Roundtable recommendations, the current study assessed the optimal therapeutic time window for PROG's neuroprotective effects when administered at 3, 6, and 24 hours post stroke in 13-month-old rats, which are better models of the human middle-aged population at greater risk for stroke than their young adult counterparts. We selected three time points to simulate realistic

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intervals when a stroke patient might reach the hospital. No other time window study of PROG treatment after brain ischemia in older animals has been reported. We evaluated PROG's effects on brain infarction using cresyl violet staining, and on functional recovery at time points from 3 to 22 days post injury using a panel of behavioral tests (gait impairment, locomotor activity, motor and grip strength, and memory impairment).

Matrix metalloproteinases (MMP) and vascular endothelial growth factor (VEGF) are associated with brain repair after stroke.[15,16] We used immunohistochemistry to investigate the effect of PROG on the vascular remodeling markers VEGF and MMP-9, and on glial fibrillary acidic protein (GFAP) as an astrocyte marker in brain tissue to illuminate some of the mechanisms of PROG's action.

MATERIALS AND METHODS

All behavioral testing, drug treatment, and histologic and immunohistochemical assays were performed independently by a researcher double-masked to the experimental conditions.

Animals and Treatment Regimen

Male Sprague–Dawley rats (450 to 500g; 13 months of age at the beginning of the experiments; Charles River Laboratories, Wilmington, MA, USA) were used. The rats were quarantined for 7 days before the experiment and housed in an AAALAC-approved Research Animal Facility with a temperature (21°C to 25°C), humidity (45% to 50%), and light-controlled environment, and placed under a 12-hour reverse light/dark cycle with free access to food and water. Public Health Service Policy on Humane Care and Use of Laboratory Animals, the Guide for the Care and Use of Laboratory Animals, and all other applicable regulations, policies, and procedures were followed and approved by the Emory University Institutional Animal Use and Care Committee (Protocol #200-1517). The experiments are reported here in accordance with the ARRIVE guidelines. Rats were randomized to the treatment conditions, and the identity of the groups was coded to avoid experimenter bias. Investigators were masked to the allocation of treatment while doing surgeries or evaluating outcomes.

A total of 86 rats underwent transient middle cerebral artery occlusion (MCAO) surgery. Six rats died during the surgical procedure and eight were excluded based on the criteria for laser Doppler flowmetry inclusion/exclusion of animals. Rats that survived transient MCAO were randomly assigned to PROG treatments given at 3 hours ($n=8$), 6 hours ($n=8$), 24 hours ($n=8$), or vehicle ($n=24$). A total of 24 animals underwent sham surgery for control groups ($n=8$ per time point). Progesterone (P-0130; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 22.5% 2-hydroxypropyl-cyclodextrin and administered intraperitoneally to ensure more rapid absorption, followed by subcutaneous injections 5 hours after the first dose and then once every 24 hours for the next 7 days. The dose was tapered by 50% over the final two treatments.

Transient Middle Cerebral Artery Occlusion

Transient cerebral ischemia was induced by occlusion of the right middle cerebral artery as previously described.[17] Our standard procedures are as follows: A midline incision was made on the ventral surface of the neck and the right common carotid arteries were isolated and ligated with 6.0 silk suture. The internal carotid and pterygopalatine artery were temporarily occluded with a microvascular clip. A 4-0 Doccol filament (Doccol Corporation, Redlands, CA, USA) was introduced into the internal carotid artery through the incision in the external carotid artery. The filament was advanced approximately 20 mm distal to the carotid bifurcation. Relative cerebral blood flow (CBF) was monitored by laser Doppler flowmetry for the entire 2 hours of occlusion. Drug treatment was randomly assigned 5 minutes before onset of reperfusion. After 2 hours of MCAO, the occluding filament was withdrawn back into the common carotid artery to allow for reperfusion. Relative CBF was then monitored for 5 minutes before the wound was sutured and the rats were then permitted to recover from anesthesia. We monitored heartbeat and blood oxygen saturation levels using a SurgiVet pulse oximeter (SurgiVet™ model V3304, Waukesha, WI, USA).

Physiological Monitoring

CBF, heart rate, hemoglobin levels, blood glucose level, and other biochemical variables were monitored continuously during surgery. Body temperature was maintained at 37°C using an automated heat lamp (Harvard Apparatus, South Natick, MA, USA). All animals that underwent stroke surgery and had laser Doppler flowmetry >40% were excluded from the study to ensure uniform and consistent large ischemic damage, thereby reducing experimental variability.[6]

Assessment of General Animal Well-Being

The animals' baseline and post-surgery weights on days 2, 7, 14, and 21 were taken as an indicator of their general well-being.

Behavioral Testing

Motor coordination. Motor impairment was assessed with the accelerating rotarod.[16] Rats were pretrained before surgery in two sessions 5 minutes apart. The animals were habituated to the stationary rod and then placed on the rotating rod. The rod was started at 2 r.p.m. and accelerated linearly to 5 r.p.m. within 180 seconds. Latency to fall off the rotarod was determined before ischemia and then post surgery. The animals were evaluated at days 2, 9, and 20 post surgery.

Grip strength. A grip strength meter (Columbus Instruments, Columbus, OH, USA) was used to measure the degree of force necessary to make the animal release a pull grid assembly with the forepaws. A digital reading (in Newtons) of two successive trials was obtained for each rat, and then averaged analysis. Baseline values for forelimb grip strength were measured presurgery and again at 3, 10, and 21 days post surgery.

Somatosensory neglect (sticky tape removal) test. This test measures the detection of, and reaction to, small pieces of adhesive tape placed on the forelimbs after MCAO. Removable sticky tape was placed on the ventral side of the animal's paw contralateral to the induced stroke. The time taken to contact/sense the tape and the time taken to remove it were recorded during a 180-second observation period. Two trials per animal were averaged for analysis. Baseline values were taken before surgery; postsurgical testing was repeated at 3, 10, and 21 days.

Spontaneous locomotor activity. Digiscan activity-monitoring boxes (AccuScan Instruments, Columbus, OH, USA) were used to assay spontaneous motor activity presurgery and then at 2, 9, and 20 days post surgery. Each session lasted 5 minutes and was conducted under red-light conditions.

Cognitive test, Morris water maze. The apparatus consists of a 133-cm diameter circular tank filled with opaque water (20 ± 1°C; Artista, Crayola, Easton, PA, USA; nontoxic white paint) to a depth of 64 cm (23 cm from top of the tank). A platform (11 × 11 cm) was submerged to a depth of 2 cm and placed approximately 28 cm from the wall of the pool in the center of the northeast quadrant. Each trial was videotaped by a ceiling-mounted video camera and the animals' movement tracked using a computer-assisted tracking system. Testing began 13 days post injury, and the rats were examined for 7 days with two trials each session. Parameters for the first test, acquisition of spatial memory, were latency to reach the platform, length of path to platform, and swim strategy, i.e., percent of total time spent in the outer versus inner annulus. The eighth session was a spatial probe trial in which the platform was removed and the rats were placed into the core of the pool and allowed to swim freely for 90 seconds. This task measures swim strategies and working (short-term, trial-to-trial) and reference (longer-term, day-to-day) memory. Time spent in the quadrant that previously contained the platform was recorded and calculated as percentage of total time spent in the pool.

Computer-assisted method for gait analysis. Animals were tested on a Catwalk system (Noldus Information Technology, Wageningen, The Netherlands), which consists of an enclosed walkway set upon on a glass plate that can be traversed by a rat from side to side. Green light enters at the long edge of the plate and can escape only where the animal's paws make contact with the glass plate. When this happens the light is scattered and the paws' images are captured by a high-speed video camera under the walkway, transformed into digital images, and transferred to a computer. Rats were pretrained on the walkway 5 days before surgery and then evaluated at postsurgical days 2, 9, and 21. After each footprint was

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identified and labeled, a wide range of gait data were generated, including: (1) the spatial parameters related to individual paws (intensity, maximum area, print area); (2) relative spatial relationship between different paws (stride length); (3) interlimb coordination (step pattern, regularity index, and phase lag); and (4) temporal parameters (swing, stance, cadence, and walk speed).

Analysis of Infarct Volume

Cerebral infarct size was evaluated using previously applied methods.[10] On postischemic day 22, animals were deeply anesthetized using isoflurane. After transcardial perfusion with cold saline followed by 10% buffered formalin, brains were extracted, fixed in gradient sucrose solution, and cut coronally into 20-μm sections for histologic analysis. On average, a total of 14 brain sections were used from each animal to evaluate infarct size. Entire brain sections were stained in 0.1% cresyl violet solution for 10 minutes at 45°C, and then rinsed in distilled water. Stained sections were fixed by serial dehydration in alcohol and xylene and mounted with xylene-based cytoseal. Fixed sections were coded to hide group identity and then scanned. The infarct areas, defined as areas showing reduced Nissl staining under light microscopy, were traced and quantified with an image analysis system. The infarct area was measured on each brain section using imaging software (ImageJ, version 1.38, NIH, Rockville, MD, USA). Infarct size was then calculated by multiplying the infarct area on each section by the distance between sections and represented as a percentage of the size of the contralateral hemisphere ± standard error of the mean (s.e.m.).

Immunohistochemistry

Slides were selected to label for VEGF, air dried for 2 hours, washed three times in phosphate-buffered saline (PBS), incubated with blocking buffer (1% bovine serum albumin in PBS) at room temperature (RT) for 30 minutes, and then incubated with rabbit polyclonal VEGF primary antibody (L1709, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at RT. The slides were then washed three times in PBS, and incubated with goat anti-rabbit immunoglobulin G labeled with Alexa Fluor 594 F(ab) fragment (A-11071, Molecular Probes, Carlsbad, CA, USA) for 1 hour at RT, rinsed with PBS for 3×5 minutes, and then covered with a mounting medium with 4′,6-diamidino-2-phenylindole. The tissue was examined with a fluorescence microscope and pictures were taken on the ipsilateral side by Image-Plus software. Vascular endothelial growth factor-positive cells were measured and calculated as a percentage of the positive area per total area by ImageJ software.

Double-immunofluorescence staining for glial fibrillary acidic protein and matrix metalloproteinase-9. Slides were selected to label for GFAP and MMP-9. The slides were air dried for 2 hours, washed three times in PBS, incubated with blocking buffer (1% bovine serum albumin in PBS) at RT for 30 minutes, and then co-incubated with rabbit anti-rat GFAP primary antibody (AB5804, Millipore, Billerica, MA, USA) and mouse anti-rat MMP-9 primary antibody (SC32791, Santa Cruz) for 1 hour at RT. The slides were then washed three times in PBS, and incubated with goat anti-rabbit immunoglobulin G (H + L) labeled with Alexa Fluor 594 F(ab) fragment (A-11071, Molecular Probes) and goat anti-mouse immunoglobulin G (H + L) labeled with Alexa Fluor 488 F(ab) fragment (A-11001, Molecular Probes) for 1 hour at RT. The slides were then rinsed with PBS for 3×5 minutes, and then covered with a mounting medium with 4′,6-diamidino-2-phenylindole. The tissue was examined with a fluorescence microscope and pictures were taken on the ipsilateral site by Image-Plus software. Glial fibrillary acidic protein-positive cells and MMP-9-positive cells were measured and calculated as a percentage of the positive area per total area by ImageJ software.

Statistical Analysis of Data

With sample sizes first determined by power analysis, repeated measures one-way analysis of variance (ANOVA) was used for behavioral experiments followed by the least significant difference and Tukey's tests for independent comparisons. Significance was set at $P<0.05$. Data are presented as mean ± s.e.m. For brain infarction data, we used unpaired *t*-tests (two-tailed).

RESULTS

Indicators of General Well-Being

Body weight of animals in the groups treated with PROG at 3 and 6 hours was restored after transient MCAO (data not shown). Repeated measures one-way ANOVA showed significant group ($P<0.001$) effects. Body weight decreased significantly ($P<0.05$) in rats subjected to MACO at 2, 9, 14, and 21 days post occlusion compared with sham rats. *Post hoc* analyses showed 3-hour and 6-hour delayed and repeated treatments with 8 mg/kg PROG given after MCAO significantly ($P<0.05$) improved body weight at 9, 14, and 21 days post surgery. We observed that the vehicle group lost significant weight and was not able to restore it compared with the PROG-treated group. In the 24-hour delayed PROG treatment group, a significant decrease in body weight ($P<0.001$) was seen at all days post surgery, and no significant improvements in weight restoration were seen.

Delayed Progesterone Treatment Improves Rotarod Performance

We observed a significant group effect in the rotarod performance of animals given delayed PROG treatment at 3 hours ($F_{(2,\ 21)}=39.68$; $P<0.001$), 6 hours ($F_{(2,\ 21)}=27.75$; $P<0.001$), and 24 hours ($F_{(2,\ 21)}=37.83$; $P<0.001$). A significant ($P<0.05$) deficit in motor performance was observed in vehicle-treated animals (under 3-, 6-, and 24-hour delayed treatment conditions) compared with their sham counterparts at 2, 9, and 20 days post surgery (Figure 1). *Post hoc* analyses showed that delayed PROG treatment at 3 and 6 hours significantly ($P<0.05$) improved the ability of animals to remain on the rotarod at all times measured, compared with their respective vehicle groups (Figures 1A and 1B). Delayed PROG treatment at 24 hours post surgery did not significantly improve motor performance at any time point compared with vehicle values (Figure 1C). However, there was a 25.6% improvement in rotarod performance by day 20.

Delayed Progesterone Treatment Improves Grip Strength

Repeated measures one-way ANOVA showed significant group effects in grip strength of animals given delayed PROG treatment at 3 hours ($F_{(2,\ 21)}=101.72$; $P<0.001$), 6 hours ($F_{(2,\ 21)}=25.58$; $P<0.001$), and 24 hours ($F_{(2,\ 21)}=19.59$; $P<0.001$). A significant ($P<0.05$) decrease in grip strength was observed in vehicle-treated animals (under 3-, 6-, and 24-hour delayed treatment conditions) compared with shams tested at 3, 9, and 21 days post surgery (Figure 1). *Post hoc* analyses showed that delayed PROG treatments at both 3 and 6 hours significantly ($P<0.05$) improved grip strength compared with vehicle counterparts at 3, 9, and 21 days post surgery (Figures 1D and 1E). We observed a 29.5% improvement in grip strength of animals treated with PROG at 24 hours post surgery compared with vehicle, but this was not statistically significant (Figure 1F).

Delayed Progesterone Treatment Improves Sensory Neglect

Repeated measures one-way ANOVA on latency to remove sticky tape from the contralateral forepaw showed significant group effects after 3-hour ($F_{(2,\ 21\ )}=\ 17.25$; $P<0.001$), 6-hour ($F_{(2,\ 21)}=39.01$; $P<0.001$), and 24-hour ($F_{(2,\ 21)}=48.80$; $P<0.001$) delayed PROG treatment. A significant ($P<0.05$) increase in latency to remove the sticker was observed in vehicle-treated animals (under 3-, 6-, and 24-hour delayed treatment conditions) compared with their respective shams at 3, 10, and 21 days post injury (Figure 2). *Post hoc* analyses showed that delayed PROG treatments at 3 and 6 hours significantly ($P<0.05$) decreased the removal latency compared with their respective vehicle values as tested at 3, 10, and 21 days post injury (Figures 2A and 2B). No significant difference in removal latency was observed in the 24-hour delayed treatment group at any time point compared with the vehicle group (Figure 2C).

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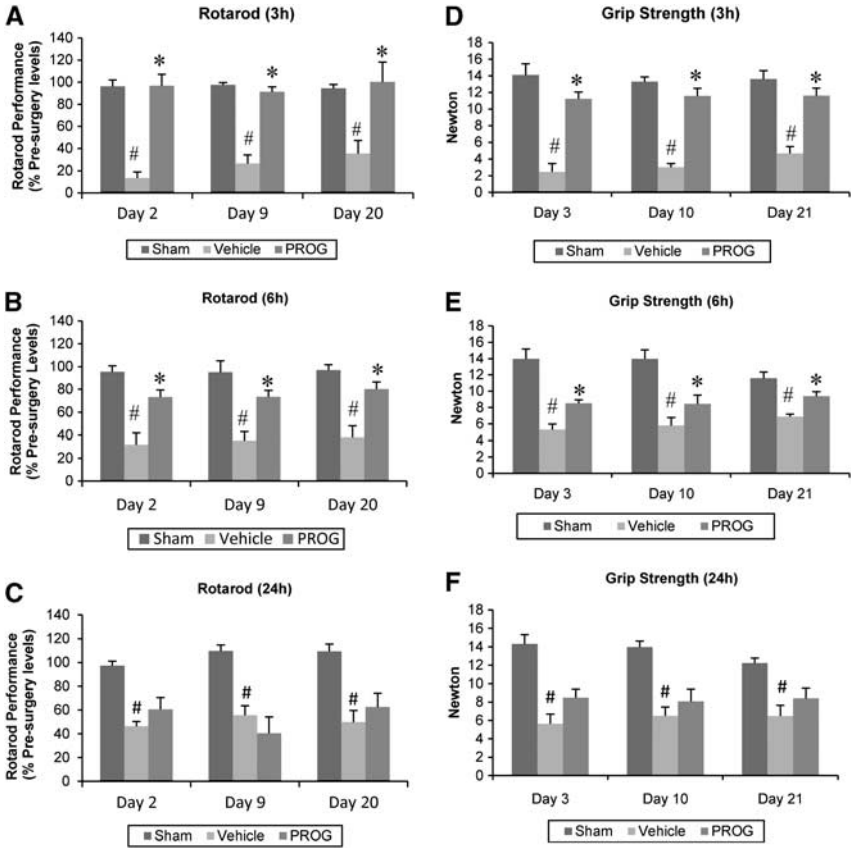

Figure 1. Effect of delayed progesterone (PROG) treatment on stroke-induced motor deficits and grip strength in middle-aged rats. Rotarod after (**A**) 3-hour, (**B**) 6-hour, and (**C**) 24-hour delayed PROG treatment. Grip strength after (**D**) 3-hour, (**E**) 6-hour, and (**F**) 24-hour delayed PROG treatment. Values are expressed as means ± s.e.m. Significant difference $^{\#}P<0.05$ compared with sham and $^{*}P<0.05$ compared with vehicle.

Delayed Progesterone Treatment Improves Locomotor Activity

Open field activity was measured at different time points after MCAO. Repeated measures one-way ANOVA on total distance traveled showed significant group effects after 3-hour ($F_{(2,\ 21)}=15.57$; $P<0.001$), 6-hour ($F_{(2,\ 21)}=12.87$; $P<0.001$), and 24-hour ($F_{(2,\ 21)}=18.92$; $P<0.003$) delayed PROG treatment. There was a significant ($P<0.05$) decrease in total distance traveled by the vehicle-treated rats (under 3-, 6-, and 24-hour delayed treatment conditions) compared with their respective controls at 2, 9, and 20 days post injury (Figure 2). *Post hoc* analyses showed that delayed PROG treatment at 3 and 6 hours significantly ($P<0.05$) increased the total distance traversed compared with their respective vehicle groups at different time points post surgery (Figures 2D and 2E). In the 24-hour delayed treatment group, PROG significantly ($P<0.05$) increased locomotor activity at day 9 post surgery compared with vehicle values. However, this improvement was transient and found to be nonsignificant (24.4%) at day 21 post surgery (Figure 2F).

Delayed Progesterone Treatment Improves Spatial Learning and Memory (Morris Water Maze)

Learning. Figure 3 shows the effects of delayed PROG treatment on mean latency (duration) to reach the hidden platform in the Morris water maze. Repeated measures one-way ANOVA on duration showed significant group effects after 3-hour ($F_{(2,\ 21)}=11.22$; $P<0.001$), 6-hour ($F_{(2,\ 21)}=15.51$; $P<0.001$), and 24-hour ($F_{(2,\ 21)}=17.22$; $P<0.001$) delayed PROG treatment. Middle cerebral artery occlusion resulted in a significant increase ($P<0.05$) in duration to reach the platform in vehicle-treated animals (under 3-, 6-, and 24-hour delayed treatment conditions) compared with their sham counterparts. Delayed PROG treatment at 3 and 6 hours, but not at 24 hours, showed a significant decrease ($P<0.05$) in duration to reach the hidden platform at all time points compared with vehicle groups (Figures 3A–3C).

Memory (time spent in platform quadrant). The probe trial revealed that vehicle-treated animals (under 3-, 6-, and 24-hour delayed treatment conditions) spent significantly ($P<0.05$) less time in the platform quadrant compared with shams. Rats treated with PROG at 3 and 6 hours post surgery spent significantly ($P<0.05$) more time in the platform quadrant compared with vehicle groups (Figures 3D and 3E). Delayed PROG treatment at 24 hours post surgery showed a 26% increase in time compared with vehicle but it was not statistically significant (Figure 3F).

Delayed Progesterone Treatment Reduces Gait Impairment

Stand or stance phase. We measured the duration in seconds of the contact of each rat's paw with the glass floor of the apparatus. Repeated measures one-way ANOVA on stand of the contralateral forepaw (% baseline) showed significant group effects after 3-hour ($F_{(2,\ 21)}=13.61$; $P=0.001$), 6-hour ($F_{(2,\ 16)}=13.05$; $P=0.001$) and 24-hour ($F_{(2,\ 21)}=17.92$; $P=0.001$) delayed PROG treatment. There was a significant ($P<0.05$) decrease in stand in vehicle-treated rats (under 3-, 6-, and 24-hour delayed treatment conditions) compared with their respective sham groups. *Post hoc* analyses showed that delayed PROG treatment at 3 and 6 hours significantly ($P<0.05$) increased stand time compared with the respective vehicle groups at 2, 9, and 21 days post injury (Figures 4A and 4B). No significant effect of 24-hour delayed PROG

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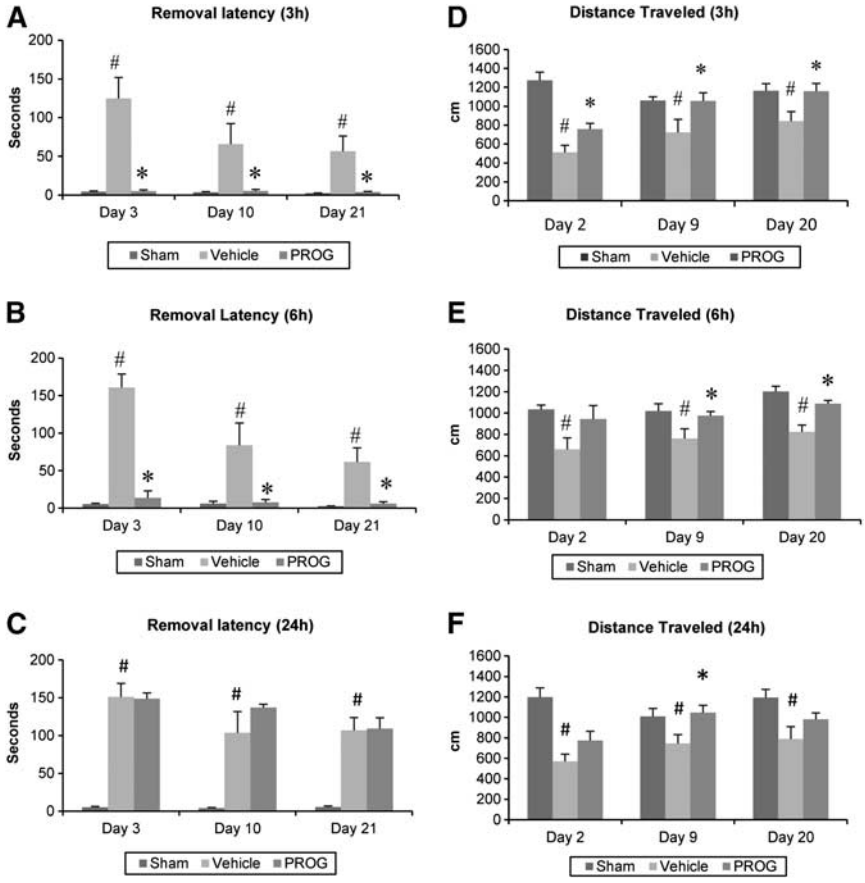

Figure 2. Effect of delayed progesterone (PROG) treatment on stroke-induced sensory-neglect deficits and locomotor impairment in middle-aged rats. Removal latency after (**A**) 3-hour, (**B**) 6-hour, and (**C**) 24-hour delayed PROG treatment. Distance traveled after (**D**) 3-hour, (**E**) 6-hour, and (**F**) 24-hour delayed PROG treatment. Values are expressed as means ± s.e.m. Significant difference $^{\#}P<0.05$ compared with sham and $^{*}P<0.05$ compared with vehicle.

treatment on stand time was observed at any time point compared with vehicle (Figure 4C).

Contact area. Contact area is a measure of spasticity. Transient MCAO led to a persistent reduction of maximal paw contact area. Repeated measures ANOVA on contact area (% baseline) of the contralateral forepaw showed a significant group effect after 3-hour ($F_{(2,\ 21)}=10.85$; $P=0.001$), 6-hour ($F_{(2,\ 21)}=2.78$; $P<0.05$), and 24-hour ($F_{(2,\ 21)}=18.27$; $P=0.001$) delayed PROG treatment. There was a significant ($P<0.05$) decrease in the contact area in vehicle-treated rats (under 3- and 24-hour delayed treatment conditions) at 2, 9, and 21 days post injury compared with their respective sham groups. *Post hoc* analyses showed that delayed PROG treatment 3 and 6 hours significantly ($P<0.05$) increased the contact area compared with their respective vehicle groups at 9 and 21 days post injury (Figures 4D and 4E). No significant effect of 24-hour delayed PROG treatment on contact area was observed at any time compared with vehicle (Figure 4F). However, we observed a 21% increase in the contact area compared with vehicle at 21 days.

Print length. This is the length (horizontal direction) of the complete paw print, which is the sum of all contacts with the floor. Repeated measures one-way ANOVA on the print length (% baseline) of the contralateral forepaw showed a significant group effect after 3-hour ($F_{(2,\ 21)}=4.05$; $P=0.032$), 6-hour ($F_{(2,\ 21)}=16.03$; $P<0.001$), and 24-hour ($F_{(2,\ 21)}=29.27$; $P<0.001$) delayed PROG treatment. We observed a significant ($P<0.05$) decrease in the print length of vehicle-treated rats (under 3-, 6-, and 24-hour delayed treatment conditions) at different times post injury compared with sham groups. *Post hoc* analyses showed that delayed PROG treatment 3 and 6 hours significantly ($P<0.05$) improved the print length compared with respective vehicle groups at 9 and 21 days post injury (Figures 4G and 4H). No significant effect of 24-hour delayed PROG treatment on print length was observed at any time compared with vehicle (Figure 4I). However, we observed a 19% increase in print length compared with vehicle at 21 days.

Delayed Progesterone Treatment Attenuates Infarction Volume

On day 21 post surgery, CV staining revealed a significant ($P<0.05$) increase in infarct volume in vehicle-treated animals (Figure 5). Delayed PROG treatment at 3 and 6 hours showed a significant ($P<0.05$) reduction in infarct volume (76.43% and 61.73%, respectively) compared with vehicle groups (Figures 5A and 5B). However, PROG treatment at 24 hours did not show any significant reduction in infarct volume compared with vehicle (Figure 5C).

Progesterone Downregulates Glial Fibrillary Acidic Protein and Upregulates Vascular Endothelial Growth Factor and Matrix Metalloproteinase-9 Expression after Stroke

We examined the expression of GFAP, a brain injury marker, and VEGF and MMP-9, markers of neovascularization, after delayed PROG treatment. A significant ($P<0.05$) increase in GFAP-positive cells was observed in vehicle groups (under 3-, 6-, and 24-hour delayed treatment conditions). Delayed PROG treatment at 3 and

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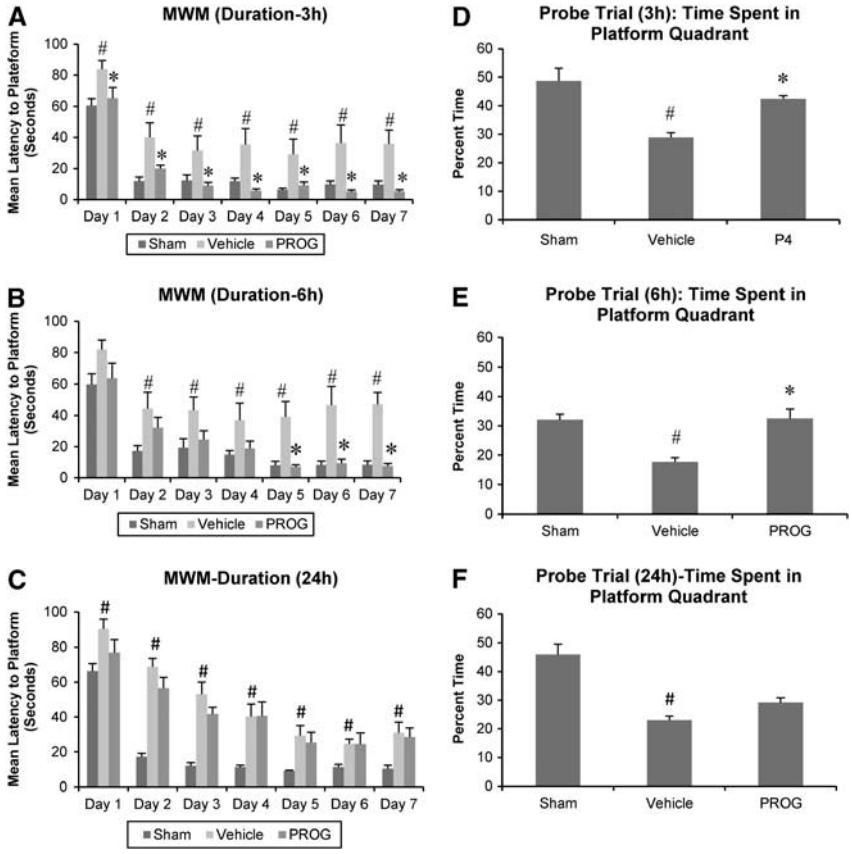

Figure 3. Effect of delayed progesterone (PROG) treatment on stroke-induced cognitive dysfunctions in middle-aged rats. Spatial learning after (**A**) 3-hour, (**B**) 6-hour, and (**C**) 24-hour delayed PROG treatment. Memory deficits (probe trial) after (**D**) 3-hour, (**E**) 6-hour, and (**F**) 24-hour delayed PROG treatment. Values are expressed as means ± s.e.m. Significant difference $^{\#}P<0.05$ compared with sham and $^{*}P<0.05$ compared with vehicle. MWM, Morris water maze.

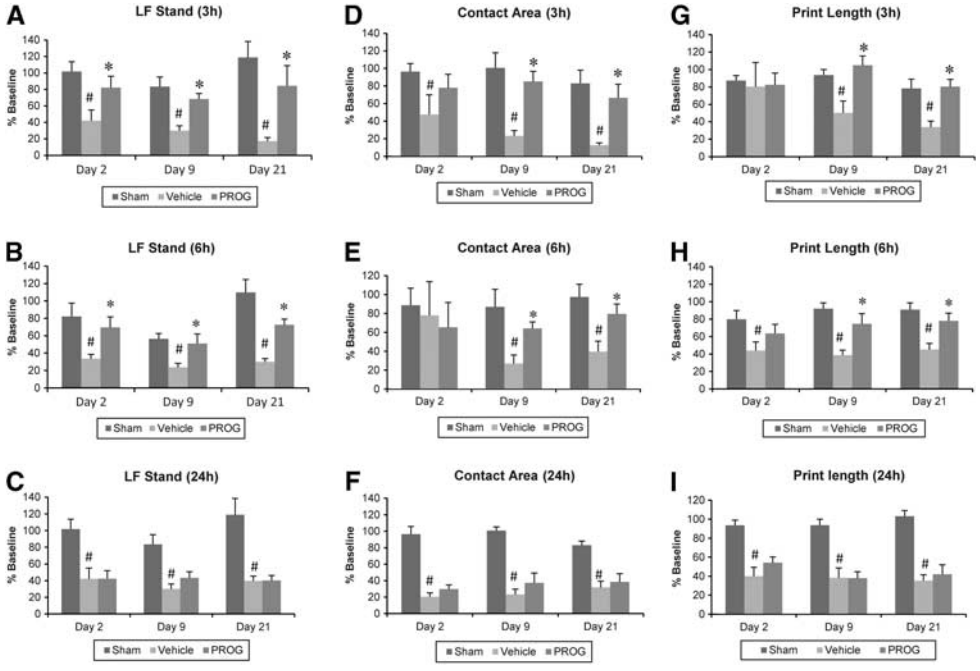

Figure 4. Effect of delayed progesterone (PROG) treatment on stroke-induced gait deficits in middle-aged rats. (**A**–**C**), stand phase; (**D**–**F**), contact area; and (**G**–**I**), print length after 3-, 6-, and 24-hour delayed PROG treatment, respectively. Values are expressed as means ± s.e.m. Significant difference $^{\#}P<0.05$ compared with sham and $^{*}P<0.05$ compared with vehicle. LF, left front.

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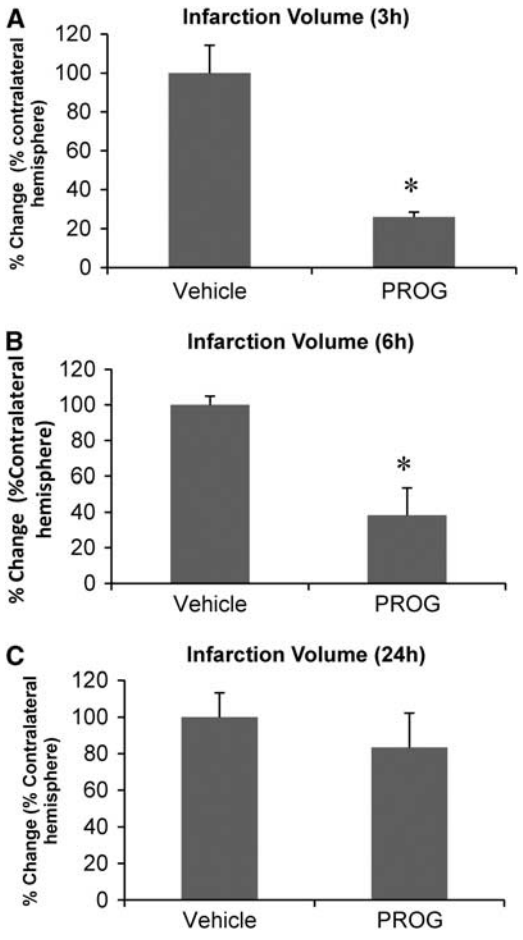

Figure 5. Effect of delayed PROG treatment on stroke-induced infarction volume in middle-aged rats. (**A**) 3-hour, (**B**) 6-hour, and (**C**) 24-hour delayed PROG treatment. Values are expressed as means ± s.e.m. Significant difference $*P < 0.05$ compared with vehicle.

6 hours, but not 24 hours, resulted in a significant decrease in GFAP-positive cells compared with their respective vehicle groups. We also observed a significant increase in VEGF- and MMP-9-positive cells in 3- and 6-hour delayed PROG treatment groups compared with their respective vehicle values, suggesting that PROG exerts a vascular remodeling effect (Figures 6 and 7). In the 24-hour delayed treatment group, PROG showed a statistically nonsignificant trend toward increase in VEGF (45.8%) and MMP-9 (54.6%), and a decrease in GFAP (25.2%) expression compared with vehicle.

DISCUSSION

The present study for the first time establishes a therapeutic time window for the neuroprotective effects of PROG against ischemic stroke-induced functional deficits and brain infarction in older male rats. The 13-month-old animals are more similar to the older human population in which stroke begins to be more prevalent.[18] We and others have shown the beneficial effects of PROG treatment given at 2 hours post occlusion after ischemic stroke,[11–14,19,20] but from a clinical point of view, it is important to determine how long PROG treatment can be delayed after reperfusion and still be effective in treating ischemic stroke. In this study, we administered PROG at 3, 6, and 24 hours post occlusion to determine the extended therapeutic time window for PROG as a treatment for ischemic stroke. We report here that PROG treatment delayed for up to 6 hours after a stroke, as measured on a battery of functional tests, can still be very effective in reducing the morphologic and behavioral impairments that accompany this injury. However, after a treatment delay of 24 hours, we observed a trend of improvement in the functional outcomes, but it was not statistically significant. Taken together with other research, our data once again highlight the importance of seeking treatment as soon as possible once the symptoms of stroke are observed.

Progesterone Reduces Sensory Impairments

Damage to cortical networks for movement control is a major cause of disability after stroke. Up to two-thirds of stroke survivors experience impaired function of the limbs[21] and the majority of the surviving patients exhibit a persistent motor disorder even after rehabilitative therapy.[22] We observed significant motor and grip deficits in vehicle-treated animals. Delayed PROG treatment at 3 and 6 hours showed marked improvement on both deficits. However, we did not observe any significant beneficial effects of 24-hour delayed PROG treatment on these deficits, although we did note a trend towards improvement.

We used the sticky tape test to evaluate the beneficial effects of delayed PROG treatment on sensory-motor impairments. Some severely ischemic animals were able to contact (sensory system) the tape at a very late phase but even after sensing the sticky paper, they were not able to remove the tape because of impairments in forelimb movement and coordination (motor system). A very significant improvement in the animals' ability to contact and remove the sticky tape was seen in both 3- and 6-hour delayed PROG treatment groups. Although we did note a trend towards improvement in the 24-hour delayed treatment group, it was not significant. Our findings are in agreement with previous reports[13,14] showing that both 3- and 6-hour delayed PROG treatment has beneficial effects on long-term functional recovery in older rats.

Progesterone Improves Memory and Spatial Learning Performance

A persistent disruption of cognitive function is often observed in stroke patients. After the stroke, we found significant cognitive impairments in the vehicle group in Morris water maze learning, and in the probe trial after 7 days of Morris water maze testing and memory formation. However, delayed PROG treatment groups at both 3 and 6 hours had significantly decreased latencies to find the hidden platform and performed significantly better than the vehicle group in the probe trial. These findings suggest that even 6-hour delayed PROG treatment can be effective in restoring cognitive functions after ischemic stroke. The present results data support previous findings from our group and others.[8,20,23]

Gait Impairments are Reduced After Delayed Progesterone Treatment

Gait impairment is also very common in stroke survivors. Several studies have reported significant gait impairments after stroke in experimental animals.[24] Vehicle-treated animals showed significant gait impairment, whereas delayed PROG treatment at 3 and 6 hours resulted in significant improvement. No significant improvement was observed in the 24-hour delayed treatment group.

Infarct Size

We and others have previously reported that PROG treatment at 2 hours post occlusion, before reperfusion, reduces infarct size in different models of stroke.[10,11,13,14,19,20] In the present study, we observed that delayed PROG treatment at 3 and 6 hours, but not

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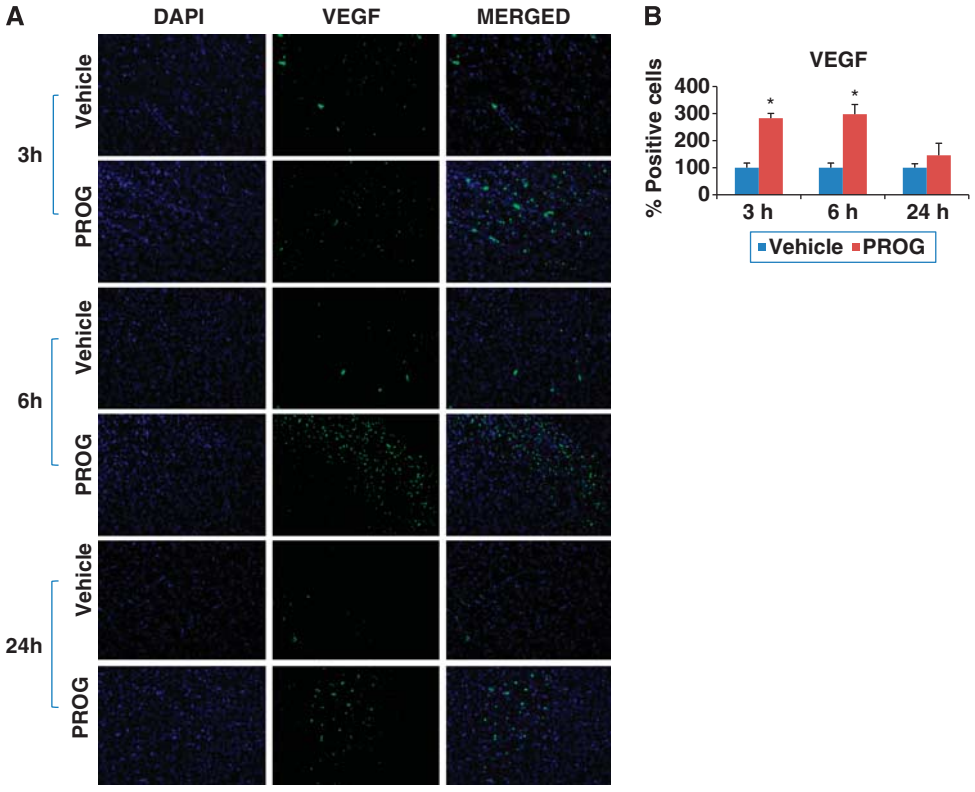

Figure 6. Progesterone downregulates vascular endothelial growth factor (VEGF) expression during the recovery phase after stroke in middle-aged rats. (**A**) Representative photomicrographs (× 40) of immunohistochemistry and (**B**) quantification of VEGF-positive cells in brain sections. Values are expressed as percent means ± s.e.m. Significant difference $^*P < 0.05$ compared with vehicle. DAPI, 4′,6-diamidino-2-phenylindole.

at 24 hours, resulted in a significant reduction in the infarction volume. These findings indicate that the time window for the potential neuroprotective effects of PROG against ischemic stroke may be limited to a range of 6 + hours after the injury.

Biomarkers of Progesterone Effects After Stroke

Having established the functional efficacy of delayed PROG treatment on stroke-induced functional deficits and brain infarction, we explored the effect of delayed PROG treatment on the expression of vascularization markers (MMP-9, VEGF) and a brain injury marker, GFAP, during the chronic/recovery phase of ischemic injury in middle-aged animals. It has been reported that PROG significantly increased VEGF protein and reduced chemokines CCL2 and CCL5 proteins after stroke.[12,25] Hwang-Levine *et al.*[26] found that allopregnanolone (a metabolite of PROG) alone increased microglial MMP-9 enzyme activity, providing a potential mechanistic basis for brain regeneration and neurovascular remodeling.

Glial fibrillary acidic protein, a major intermediate filament in mature astrocytes, is important for astrocyte–neuronal interactions and has a vital role in modulating synaptic efficacy in the central nervous system as well as in brain plasticity.[27] After stroke, GFAP expression increases in astrocytes, and activated astrocytes release cytokines, upregulate iNOS, and impede functional recovery as a result of this inflammatory cascade.[28,29] We observed a significant increase in GFAP-positive cells in the vehicle group, indicating a strong astrocyte response to ischemic injury that could lead to scar tissue formation which, in turn, could impede functional and structural recovery. Interestingly, delayed PROG treatment at both 3 and 6 hours, but not 24 hours, showed less GFAP expression. Our results agree with previous findings where, in a TBI model, PROG treatment reduced GFAP expression.[30]

Vascular endothelial growth factor, an important neurotrophic molecule, is naturally expressed in the brain and upregulated by hypoxia.[16,31–33] It is thus part of an endogenous adaptive system to protect the brain from ischemia.[32] Vascular endothelial growth factor upregulation during the chronic phase of stroke has been reported to improve functional outcomes and decrease brain injury by protecting injured neurons after injury.[15,32–36]

During the acute phase of stroke, activation of matrix metalloproteinases is believed to be harmful because these molecules contribute to the injury by disrupting the neurovascular unit, and their upregulation leads to cell matrix homeostasis disruption, blood–brain barrier damage, vasogenic edema formation, an increase in brain infarction, and hemorrhagic transformation.[36] However, it has been suggested that both VEGF and matrix metalloproteinases can also have beneficial roles in the chronic/recovery phase of stroke and in neurovascular remodeling as the brain seeks to heal itself after injury.[15,37] Our data show that delayed PROG treatment at 3 and 6 hours increased the expression of VEGF and MMP-9 as evidenced by immunohistochemistry immunostaining in brain sections at 3 weeks after stroke. These findings suggest that VEGF and MMP-9 could have a role in the neuroprotective effects of PROG during the chronic phase of the disease.[38]

Interestingly, we did not observe any significant change in the expression of MMP-9 or VEGF in 24-hour delayed PROG-treated groups compared with vehicle, suggesting that the time of PROG administration is critical. The exact mechanism of PROG-induced MMP-9 and VEGF activation during the recovery phase of ischemic injury when administered at 3 and 6 hours, but not 24 hours, post injury remains to be explored.

In conclusion, our data suggest that delayed PROG treatment up to 6 hours post stroke has the potential to protect brain against

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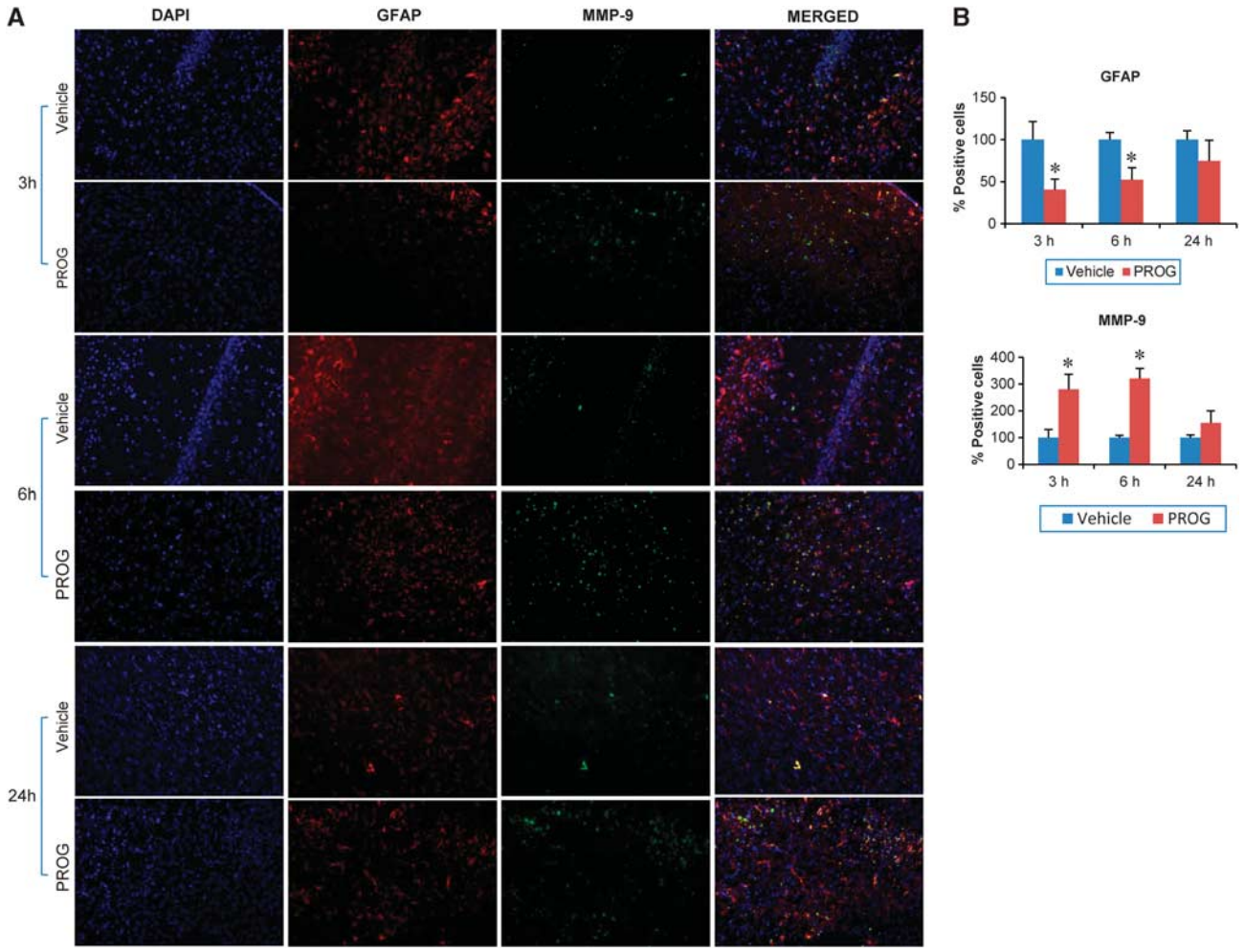

Figure 7. Progesterone upregulates glial fibrillary acidic protein (GFAP) and matrix metalloproteinase-9 (MMP-9) expression during the recovery phase after stroke in middle-aged rats. (**A**) Representative photomicrographs ($\times$ 40) of double immunostatining for (glial fibrillary acidic protein) GFAP and MMP-9-positve cells in different groups after 3-, 6-, and 24-hour delayed PROG treatment. (**B**) Quantification of GFAP and MMP-9-positve cells in brain sections. Values are expressed as percent means $\pm$ s.e.m. Significant difference $^*P < 0.05$ compared with vehicle. DAPI, 4′,6-diamidino-2-phenylindole.

ischemic/reperfusion damage at both morphologic and behavioral levels by modulating the activity of VEGF and MMP-9 at the delayed/recovery phase of injury. The data are clinically relevant, because they provide further evidence that PROG can be a safe and effective therapeutic drug for ischemic stroke, with a relatively extended therapeutic time window. It is important to note that this window appears to close by 24 hours post injury, so although PROG treatment is beneficial for at least 6 or more hours after a stroke, the opportunity to attenuate the injury cascade is not unlimited. This emphasizes again why it is so critical for patients to recognize the early symptoms of stroke and seek treatment as soon as possible.

Limitations and Future Directions

This study was conducted in male subjects only. Progesterone has already been shown to be neuroprotective in aged and ovariectomized female mice after transient stroke.[19] However, it would be interesting to compare the beneficial effects of delayed PROG treatment in aged male and female animals with effects in their younger counterparts. A meta-analysis of preclinical data using PROG in females with experimental stroke suggests that there was an increase in the incidence of stroke-related death in adult, overiectomized females. These findings highlight the fact that further investigations are needed to evaluate the hormonal status of females at the time of injury as well as dose– and time–response relationships.[39]A recent clinical report used data from the National Trauma Data Bank to examine the influence of gender on in-hospital mortality after TBI in over 20,000 teenagers.[40] The authors concluded that pubescent females had significantly reduced mortality rates compared with males of comparable age.

In future studies, it would be interesting to explore the therapeutic time window of PROG's efficacy if administered between 6 and 24 hours post stroke—e.g., with an 8- or 12-hour window. Given the trends we observed at 24 hours, it might also be worthwhile in a future study to examine whether larger or more frequent doses of PROG, given later in the injury cascade, might also prove to have some efficacy, especially in older subjects who are more at risk for stroke.

DISCLOSURE/CONFLICT OF INTEREST

DG Stein is entitled to royalties from products of BHR Pharma LLC (BHR) related to the use of PROG in TBI and stroke, and receives research funding from BHR, which is developing products related to this research. In addition, he serves as a consultant to BHR and receives compensation for these services. The terms of this arrangement have been reviewed and approved by Emory University, which receives the largest

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share of fees in accordance with its conflict of interest policies. The remaining authors declare no conflict of interest.

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