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Progesterone improves long-term functional and histological outcomes after permanent stroke in older rats

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Abstract

Previous studies have shown progesterone to be beneficial in animal models of central nervous system injury, but less is known about its longer-term sustained effects on recovery of function following stroke. We evaluated progesterone's effects on a panel of behavioral tests up to 8 weeks after permanent middle cerebral artery occlusion (pMCAO). Male Sprague-Dawley rats 12 m.o. were subjected to pMCAO and, beginning 3 h post-pMCAO, given intraperitoneal injections of progesterone (8 mg/kg) or vehicle, followed by subcutaneous injections at 8 h and then every 24 h for 7 days, with tapering of the last 2 treatments. The rats were then tested on functional recovery at 3, 6 and 8 weeks post-stroke. We observed that progesterone-treated animals showed attenuation of infarct volume and improved functional outcomes at 8 weeks after stroke on grip strength, sensory neglect, motor coordination and spatial navigation tests. Progesterone treatments significantly improved motor deficits in the affected limb on a number of gait parameters. Glial fibrillary acidic protein expression was increased in the vehicle group and considerably lowered in the progesterone group at 8 weeks post-stroke. With repeated post-stroke testing, sensory neglect and some aspects of spatial learning performance showed spontaneous recovery, but on gait and grip-strength measures progesterone given only in the acute stage of stroke (first 7 days) showed sustained beneficial effects on all other measures of functional recovery up to 8 weeks post-stroke.

Keywords

Functional recovery; Long-term functional deficit; Permanent stroke; Progesterone; Stroke

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AUTHORS' CONTRIBUTIONS BW contributed to the manuscript draft, experimental design and execution, data analysis and generation of figures; TI performed the surgeries; DGS conceived the study, and contributed to the study design and the writing of the manuscript. IS contributed to the study design, data analysis and interpretation, and wrote and edited the manuscript.

CONFLICT OF INTEREST DGS and IS along with Emory University retain patents related to the use of progesterone in TBI and certain forms of CNS tumors but have no financial gains, royalties or licensing agreements from research on progesterone. BW and TI declare no conflict of interest.

1.0 INTRODUCTION

Numerous drugs have shown promise in preclinical animal models of stroke, but none have proven effective in clinical trials. There are now a number of papers suggesting that pre-clinical studies do not provide enough information about whether potential treatments are effective in clinically relevant models of stroke in older subjects, or whether the treatment effects are long-lasting [1]. The study of a neuroprotective agent after a brief recovery may not predict final outcome. For example, interventions such as sigma receptor agonist 1,3-di-o-tolylguanidine [2], ischemic preconditioning [3], hypothermia [4], α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)1 antagonist NBQX [5], N-type calcium channel antagonist Ziconotide (SNX-111), and N-Methyl-D-aspartate (NMDA) antagonist MK-801 [6] afford only transient functional benefits and failed to show sustained efficacy.

Despite a substantial body of experimental preclinical stroke work from our laboratory [7–15] and others [16–24] documenting the neuroprotective effects of progesterone, two recently completed phase III clinical trials found that progesterone was not helpful to patients recovering from moderate to severe traumatic brain injury (TBI) [25,26]. Although the authors of the two independent trials cite a number of potentially confounding factors, the reasons for the trials' negative results remain to be fully determined [27,28]. Nonetheless, the outcomes make it clear that greater scrutiny of preclinical work will be required before progesterone is tested in other clinical conditions including stroke. Evaluating stroke parameters in more clinically relevant animal models will provide data that can translate more effectively to stroke in humans.

In a systematic dose-response and time-window study in rats, we previously showed that an 8-mg/kg dose of progesterone can be given up to 6 h after stroke onset with significant improvement in motor, sensory and memory function measured 3 weeks after ischemic stroke [8]. While this represents a potentially important step in developing a safe and effective neuroprotective treatment for stroke, it is also important to determine whether the beneficial effects of progesterone are sustained well after treatments have terminated. To better simulate the typical human stroke injury, the Stroke Treatment Academic Industry Roundtable (STAIR) endorses the testing of agents in permanent models of ischemia [29,30]. It has been proposed that because of shorter treatment/response duration, permanent or gradually reversed vascular occlusion is a more clinically relevant experimental stroke model for therapeutic predictions than the transient occlusion model because it may respond to treatment for considerably longer durations [31].

Although stroke is a disease of elderly, most animal stroke studies are done in younger subjects. Older patients have higher in-hospital mortality as well as poorer functional outcomes after an ischemic event [32]. Further, there has been a significant increase of stroke cases in middle-aged, often obese, people [33]. A recent American Heart Association

¹**Abbreviations:** AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA: analysis of variance; CCA: common carotid artery; GFAP: glial fibrillary acidic protein; HBC: 2-hydroxypropyl- β -cyclodextrin; LSD: least significant difference; MCA: middle cerebral artery; MWM: Morris Water Maze; NBQX: N-type calcium channel antagonist; NMDA: N-Methyl-D-aspartate; PBS: phosphate-buffered saline; pMCAO: permanent middle cerebral artery occlusion; PROG: progesterone; RT: room temperature; SNX-111: Ziconotide; STAIR: Stroke Treatment Academic Industry Roundtable; TBI: traumatic brain injury

report shows sharp increases in midlife stroke cases [34]. Recommendations from experts and established guidelines [35] on how to conduct preclinical research to identify clinical candidates suggest that, to improve the utility of animal stroke models, infarcts should be produced in older animals, and potential efficacy should be determined on the basis of behavioral/functional effects after longer survival times [35,36]. Because of these concerns, for longer-term studies, we used a permanent middle cerebral artery occlusion (pMCAO) model of ischemia in older rats. We evaluated the effects of progesterone administration with a clinically relevant delay of 3 h post-stroke on a panel of quantitative, functional and behavioral tests at 3, 6 and 8 weeks post-pMCAO.

2.0 MATERIALS AND METHODS

2.1 Experimental Design

The experiments reported here comply with the Rigorous Study Design and Transparent Reporting (RIGOR) guidelines [37,38]. Twenty-nine 12-month-old virgin male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) underwent pMCAO by electrocoagulation, or sham operation. Based on a delta-value of 1.5 we calculated the sample sizes and power needed to reject the null hypothesis (of no differences among the injured/pMCAO group relative to controls) to achieve 80% power to detect a 50% difference between treatment and controls. We calculated the starting sample sizes to be at least 8 animals/group. The animals were housed in individual cages with free access to pellet chow and water, quarantined for one week, and handled at least 5 times before starting training. Room temperature was maintained at 21–25° C, and humidity at 45–50%. Rats were maintained under a 12:12-h reverse light-dark cycle (0900–2100 h) so that behavioral testing would occur during their active phase. 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 #2001517). Rats were randomized to the treatment conditions, and the identity of the groups was coded to avoid experimenter bias. Daily treatment allocation (vehicle vs. progesterone) was coded and randomly determined by one investigator (IS). Another investigator (TI) blinded to treatment independently performed surgeries, but did not participate in outcome evaluations. Preclinical outcomes (mortality, behavioral tests, histological analysis) were determined by a third investigator (BW) who was blinded to the treatment.

After pMCAO or sham operation, animals were randomly assigned to one of three groups: sham+vehicle (n= 9), pMCAO+vehicle (n=10), and pMCAO+progesterone (PROG) (n=10). Three hours post-pMCAO, all animals received an intraperitoneal injection of either 22.5% 2-hydroxypropyl- β -cyclodextrin (HBC; Sigma-Aldrich, St. Louis, MO, USA) or PROG (8 mg/kg; Sigma-Aldrich), followed by subcutaneous injections 5 h later, and then every 24 h for the next 7 days. The last two injections were tapered in order to wean the animals off the treatment without side effects caused by abrupt withdrawal of the hormone [39]. Thus, after 5 consecutive days of 8 mg/kg doses, rats received 4 mg/kg on the 6th day and 2 mg/kg on the 7th [39]. Each dose of HBC and progesterone was administered in equal volumes relative to body weight. Rats were perfused 8 weeks post-stroke after completing behavioral

testing and their brains were cryoprotected for infarct volume measurements. One rat each in the vehicle and progesterone groups died at 10 and 15 days post-pMCAO, respectively, and their data were not included in the final analyses.

2.2 Induction of permanent focal cerebral ischemia

We used a permanent stroke model using direct ligation (cauterization) of the MCA in rats as developed by Tamura *et al.* [40] Tamura *et al.* and modified by Chen *et al.* [41]. All rats were anesthetized using 5% isoflurane and maintained at 1.5–2% (2:1 nitrous oxide and oxygen) during surgery. The incision area was shaved and sterilized with Betadine® antiseptic and 70% isopropanol. A ventral midline incision was made for exposure of both common carotid arteries (CCA). The contralateral right CCA was permanently ligated using a 3/0 silk suture, and the left ipsilateral CCA was temporarily occluded for 90 min using a Mayfield micro-aneurysm clip. A vertical incision was made midway between the left orbit and the left external auditory canal. The left temporalis muscles were separated and retracted inferiorly downward to expose the zygomatic and squamosal bone, and the pterygoid muscles and mandibular nerve were then retracted to expose the ventral surface of the skull. Under an operating microscope, the bone around the *foramen ovale* was burnished (2–3 mm) to expose the MCA, and the craniotomy was extended dorsally up to the first major branch of the MCA. Care was taken to avoid thermal and physical injury to the cortex during preparation for exposing the MCA. The dura was opened with a bent 26-gauge needle, the arachnoid membrane was gently removed and the MCA cauterized and cut permanently to prevent recanalization with a bipolar electrocauterizer without damaging the brain surface. The site of the occlusion is midway between the inferior cerebral vein and olfactory tract. Sutures were used to close the incision after bleeding stopped. The rats were allowed to recover from anesthesia on the heating pad and then returned to their home cages. After 90 min of temporary occlusion, the micro-aneurysm clip was removed from the left ipsilateral side and rats were again permitted to recover from anesthesia on the heating pad.

Sham-operated rats were subjected only to exposure of the MCA without coagulation. Physiological parameters were monitored with pulse oximetry (model V3304, SurgiVet™, Waukesha, WI, USA): heart rate was maintained around 350 beats per minute and blood oxygen level (SpO₂) was kept above 95%. Core body temperature ($37 \pm 2^\circ \text{C}$) was maintained with a homeothermic heating blanket system (Harvard Apparatus, Holliston, MA, USA).

2.3 Assessment of functional outcome

At repeated intervals (3, 6 and 8 weeks post-pMCAO), behavioral recovery was evaluated for grip strength (20, 40, and 53 days post-pMCAO), motor ability to remain on an accelerating rotarod (21, 41, and 54 days post-pMCAO), sensory neglect (21, 41, and 54 days post-pMCAO), gait impairment (20, 40, and 53 days post-pMCAO), and learning and memory in a spatial navigation task (starting at day 44). Throughout the daily injections, behavioral testing, and histological analysis, experimenters were blinded to the experimental conditions of the animals.

2.3.1 Grip Strength—To measure the rats' forelimb grip strength, we used an electronic digital force gauge grip-strength meter (Columbus Instruments, Columbus, OH, USA) that recorded the peak force exerted by the animal while gripping the sensor bar as previously described [12]. A digital reading (in Newtons) of three successive trials was obtained for each rat, and the best (highest) score was used for data analysis. Baseline was determined one day prior to ischemia and animals were tested at 3, 6 and 8 weeks post-ischemia.

2.3.2 Motor Impairment—Motor impairment was assessed with an accelerating rotarod (Columbus Instruments). Rats were given 5 training sessions 5 min apart before establishing baseline as described previously [12]. Two trials were given to each animal with 5 min of rest time between trials, and the best (longest) latency values were used for analysis. Latency to fall off the rotarod was determined before surgery and at 3, 6 and 8 weeks post-surgery.

2.3.3 Somatosensory neglect—An adhesive removal test was performed as previously described [8] to evaluate somatosensory function. Habituation and testing occurred under red light. Half-inch round adhesive labels were placed on the ventral surface of the contralateral forepaw. The experimenter recorded the latency for each rat to remove the adhesive label with its mouth up to a maximum latency of 2 min. Two trials were given to each animal with 5 min rest time between trials, and the best (shortest) latency values were used for analysis. Latency to remove the sticker was recorded one day before surgery and at 3, 6 and 8 weeks post- injury.

2.3.4 Spatial navigation—A spatial learning and memory test using the Morris water maze (MWM) was conducted as previously described [42]. Briefly, the animal's position in the maze and latency to reach platform was recorded and analyzed using a computer-assisted tracking system (Clev Sys Inc., Reston, VA). Beginning at 44 days post-pMCAO, all rats were tested for acquisition in the MWM for 5 consecutive days. Each animal received 2 trials per day, separated by a 5-min interval. The rats were placed in the pool and allowed to swim until they reached the platform or until 90 s had elapsed. When rats were unable to locate the MWM platform within 90 s, the experimenter guided the rats to the platform, where they were allowed to stay for 20 s before being removed. After 5 min, subjects were again released into the tank from the adjacent quadrant of the tank and allowed to swim to the platform. After 5 consecutive days of acquisition trials, there was a 2-day break, and then the probe test was conducted at day 51 post-injury. For this trial, the hidden platform was removed and rats were placed in the pool to swim for 60 s starting from the same location as in the acquisition tests. The time spent swimming in the quadrant where the platform had been located was recorded.

2.3.5 Gait analysis—The CatWalk (Noldus Information Technology, Wageningen, The Netherlands), a quantitative gait analysis system, was used for assessment of gait-related anomalies following stroke [43,44]. A fluorescent light is reflected internally in the glass floor of the walkway when the animal crosses the walkway. The video images with paw prints were classified by assigning labels (e.g., right and left, fore and hind paw) automatically. The experiment was performed in red light. Before surgery all animals were trained to traverse a glass walkway toward their home cages from one end to another without

interruptions, so that all the animals started from more or less the same baseline. On subsequent training days, 3 complete runs across the walkway were recorded. If an animal failed to complete a run, walked backwards, or reared during the run, it was given an additional run.

2.4 Assessment of infarct volume

Cerebral infarct size was assessed according to previously applied methods [11]. The brains were embedded in OCT (optimal cutting temperature compound) and sectioned coronally in 20- μ m thick slices. Six evenly spaced intermediate sections starting from the frontal pole at an interval of 1 mm were used for infarct measurements. Cresyl violet-stained slides were scanned using Silverfast Pathscan software (PathScan Enabler IV, Meyer Instruments, Houston TX). The infarct areas, defined as areas showing reduced Nissl staining under light microscopy, were traced and quantified using imaging software (ImageJ 1.38, NIH, Rockville, MD, USA). The area of infarction was determined by averaging the percentage of the damaged tissue across the selected sections, and then expressed as a percentage of the intact contralateral side $\pm$ SEM. These techniques have been used repeatedly in the literature to measure and evaluate stroke outcome in experimental preparations [8,11,45].

2.5 Immunohistochemistry

Histologic cryosections (20 μ m in thickness) were selected for immunohistochemical analysis and immunolabeled for glial fibrillary acidic protein (GFAP) and Iba1. Sections were air-dried for 1 h and washed in 1x phosphate buffer saline (PBS) 5 times, then incubated in with protein blocking serum at room temperature (RT) for 1 h. Sections were then incubated with primary antibody GFAP (1:50, Millipore, Billarica, MA, USA) and Iba1 (1:1000, Wako, Richmond, VA, USA) for 1 h at RT. and then washed with 1X PBS 5 times followed by incubation with secondary goat anti mouse (1:100, Alexa Fluor 488 IgG (H+L), A11001, Invitrogen, Carlsbad, CA, USA) for 30 min at RT. Slides were washed with 1 X PBS 5 times and covered with mounting medium (Vectashield). The slides were examined under a fluorescence microscope and pictures were taken from the peri-infarct area using a digital camera mounted on Olympus microscope (Olympus BX41, Olympus America Inc.).

2.6 Data analysis

All results were expressed as mean $\pm$ standard error of the mean and calculations were obtained using SPSS 21.0 software. All behavioral data except those for the spatial memory (probe) trial were analyzed by repeated-measures analysis of variance (ANOVA) followed by a Tukey's *post-hoc* test. Other results including infarction volume and the probe trial were analyzed with oneway ANOVA followed by LSD and Tukey HSD post-test for multiple comparisons. The criterion for statistical significance was set at $p < 0.05$.

3.0 RESULTS

3.1 Behavioral Analysis

3.1.1 Grip Strength—Progesterone treatment produced sustained grip strength improvement following pMCAO (Fig. 1A). There was a significant group ($F_{(2,24)} = 41.61$, $p < 0.001$) effect. Grip strength decreased significantly ($p < 0.05$) in stroked rats (12.00 ± 0.42 ,

11.61 $\pm$ 0.51, and 11.68 $\pm$ 0.39 Newtons at 3, 6 and 8 weeks post-occlusion, respectively) at all time points compared to intact rats (17.28 $\pm$ 0.49, 16.39 $\pm$ 0.70, and 16.09 $\pm$ 0.67 Newtons). *Post-hoc* analyses showed 3-h delayed and repeated treatments with progesterone given after pMCAO significantly ($p < 0.001$) improved grip strength at 3 and 8 weeks following injury (14.35 $\pm$ 0.57, 13.19 $\pm$ 0.33, and 14.30 $\pm$ 0.47, respectively). A deficit in motor strength of 27.44% compared to shams persisted at 8 weeks post-occlusion.

3.1.2 Rotarod—The measures of latency to remain on the rotarod showed significant group differences ($F_{(2,24)} = 11.96$, $p < 0.001$). There were significant ($p < 0.001$) deficits (Fig. 1B) in motor performance (time spent on the rotarod) in pMCAO+vehicle-treated rats (58.39 $\pm$ 5.77, 41.59 $\pm$ 7.33, 35.15 $\pm$ 5.04 at 3, 6 and 8 weeks post-occlusion) compared to shams (90.33 $\pm$ 5.20, 68.22 $\pm$ 2.72, 77.90 $\pm$ 6.63). *Post-hoc* analyses found that repeated treatments with progesterone after pMCAO significantly ($p < 0.001$) improved ability to remain on the rotarod (89.23 $\pm$ 10.35, 55.94 $\pm$ 8.74, 69.69 $\pm$ 8.52) at 3 and 8 weeks post-occlusion. A substantial deficit (54.87% compared to shams) in motor ability persisted at 8 weeks post-occlusion. Progesterone-mediated improvement (80.79% compared to the pMCAO+vehicle-treated group) was sustained up to 8 weeks post-surgery.

3.3.3 Sensory Neglect—Measures of latency to remove sticky tape from the contralateral forepaw showed significant group ($F_{(2,23)} = 5.98$) effects. There was a significant ($p < 0.05$) increase in latency (sec) to remove the stickers in pMCAO+vehicle-treated rats (42.50 $\pm$ 11.01, 35.87 $\pm$ 10.63, and 24.87 $\pm$ 5.57 at 3, 6 and 8 weeks post-occlusion, respectively) compared to shams (10.33 $\pm$ 1.51, 10.66 $\pm$ 1.51, and 8.00 $\pm$ 1.65) (Fig. 1C). A 3-h delay of progesterone treatment after pMCAO ($p < 0.05$) decreased latency to remove the sticker (19.88 $\pm$ 8.28, 13.11 $\pm$ 3.18, and 19.33 $\pm$ 6.47) at 3, 6 and 8 weeks. This decrease was observed to be significant at 3 and 6 weeks only. Latency to remove the stickers in pMCAO +vehicle-treated rats improved over post-occlusion time, contributing to the non-significant improvement observed with progesterone at week 8 post-occlusion.

3.3.4 Spatial learning and memory

3.3.4.1 Learning: Fig. 1D and E shows the effects of delayed progesterone treatment on the mean latency (duration) to reach the hidden platform in the MWM. Repeated measures ANOVA on duration showed significant group effects ($F_{(2,24)} = 3.91$, $p < 0.05$) in trial 2 (working memory). Over 5 consecutive days, sham and progesterone-treated animals showed progressive reduction in latency to find the platform, whereas the stroke+vehicle-treated group showed a slower decline in latency that became significant at days 3 and 5. No significant group effect ($F_{(2,24)} = 1.46$, $p < 0.05$) was observed in the latency to find the platform in run 1 (reference memory).

3.3.4.2 Memory: On the probe trial significant difference among the treatment groups ($F_{(2,24)} = 4.69$, $p < 0.05$) was observed. *Post-hoc* analyses showed that subjects in the stroke +vehicle-treated group spent significantly less time (27.47 $\pm$ 1.69%) in the platform quadrant compared to the sham+vehicle-treated group (40.28 $\pm$ 3.52%) (Fig. 1F). Progesterone treatment with a 3-h delay (33.52 $\pm$ 9.97%) after pMCAO increased the time spent in

platform quadrant but *post-hoc* analysis showed that this increase (47.24% improvement) was not significantly different from that of the pMCAO-vehicle group.

3.3.5 Gait Impairment

3.3.5.1 Stride length: Stride length is the distance between successive placements of the same paw and is a measure of normal performance. Following pMCAO, stride length significantly decreased in the affected forepaw (Fig. 2A). Progesterone treatment improved forepaw stride length of the affected (contralateral) limb. The measures of stride length (% baseline) of the contralateral forepaw showed significant group ($F_{(2,24)} = 16.90$, $p < 0.05$) effects. There was a significant decrease in stride length in pMCAO+vehicle-treated rats (81.35 ± 6.64 , 81.39 ± 6.65 , 77.19 ± 8.47 at 3, 6 and 8 weeks post-occlusion, respectively) compared to shams (139.56 ± 14.63 , 136.85 ± 10.43 , 143.00 ± 15.15). Progesterone-treated rats showed improvement (109.67 ± 3.89 , 106.14 ± 4.57 , 105.99 ± 4.75 at 3, 6 and 8 weeks post-occlusion, respectively) compared to the pMCAO-vehicle group ($p < 0.05$).

3.3.5.2 Paw print area: Paw print area is a measure of spasticity. Permanent MCAO led to a persistent reduction of maximal paw contact area with the runway. Measures of the paw print area (% baseline) of the contralateral forepaw showed significant group ($F_{(2,24)} = 4.44$, $p < 0.05$) effects. The print area at maximal contact of contralateral forepaws (right paw) of rats during floor contact in pMCAO+vehicle-treated rats was significantly smaller (59.78 ± 12.14 , 66.82 ± 8.81 , 70.71 ± 8.03 at 3, 6 and 8 weeks post-occlusion, respectively) compared to sham rats (91.40 ± 8.81 , 97.66 ± 7.72 , 102.11 ± 7.96) (Fig. 2B). All progesterone-treated rats showed greater contact area (82.82 ± 7.98 , 88.53 ± 9.61 , 83.47 ± 11.51 at 3, 6 and 8 weeks post-occlusion, respectively) with the contralateral forelimb ($p < 0.05$).

3.3.5.3 Swing speed: Swing speed is the speed (distance unit/second) of the paw during swing and is a measure of normal gait. Swing speed significantly decreased in the affected forepaw at all the time points measured following pMCAO (Fig. 2C). Measures of swing speed (% baseline) of the contralateral forepaw showed significant group ($F_{(2,24)} = 14.48$, $p < 0.05$) effects. There was a significant decrease in the swing speed in pMCAO+vehicle-treated rats (71.01 ± 4.69 , 77.49 ± 7.46 , 76.16 ± 8.72 at 3, 6 and 8 weeks post-occlusion, respectively) compared to shams (113.75 ± 4.39 , 119.12 ± 8.28 , 116.40 ± 8.22) (Fig. 2C). The progesterone-treated rats showed improvement (96.31 ± 11.33 , 104.39 ± 7.06 , 96.48 ± 6.91) compared to the pMCAO-vehicle group ($p < 0.05$).

3.4 Infarction volume

ANOVA showed a difference among the treatment groups ($F_{(1, 14)} = 7.5$, $p < 0.05$). Three-hour delayed progesterone treatment produced a significant reduction of 62.56 % in the volume of the infarct (% contralateral hemisphere) measured at 8 weeks post-occlusion compared to the stroke+vehicle-only group (4.07 ± 0.34 vs. 10.87 ± 2.46) (Fig. 3).

3.5 Gliosis

The immunohistochemical assay showed a significant increase in astrogliosis (GFAP immunoreactivity) and microgliosis (Iba1 immunoreactivity) in the brain tissues of the

vehicle group. Progesterone treatment markedly decreased the intensity of GFAP (Fig. 4) and Iba1 (Fig. 5) at 8 weeks post-injury compared to vehicle ($p<0.05$).

4.0 DISCUSSION

Despite the fact that injuries to the brain caused by ischemic stroke continue to evolve weeks to months after the stroke, most pre-clinical neuroprotective studies are limited by the use of short survival times and the lack of long-term assessment of cell death and its functional consequences [46]. In our study, we observed that, compared to intact rats, our mature rats with pMCAO showed sustained sensory, motor, and gait impairments and some cognitive deficits over 8 weeks of post-stroke testing.

In pre-clinical models, extended testing can determine whether the beneficial effects of a pharmacologic intervention are sustained or simply delay the appearance of a deficit [47,48] long after the treatment is discontinued. Most studies report that progesterone is effective in acute reduction of lesion size and in the improvement of early behavioral impairments after stroke, but less is known about its long-term neuroprotective effects. Our previous research in both permanent and transient models of ischemic stroke [8,9,49] evaluated dose-response and therapeutic time window parameters of the effects of progesterone treatment on functional and histological outcome measures at a survival time of 3 weeks. For the present study we used a longer survival time of 8 weeks even though treatment was terminated 7 days after the stroke. Our current finding of persistent histologic and functional protection in a clinically relevant model of permanent stroke is an important advance over previous efficacy studies, especially given that progesterone administration was delayed 3 h after the onset of stroke. This study used direct ligation (cauterization) of the MCA in rats as developed by Tamura et al. [40] and modified by Chen *et al.* [41]. By interrupting the blood supply (collateral circulation) to the right MCA territory by permanently ligating the right CCA and transiently occluding the left CC with a clip, Chen *et al.* were able to consistently produce a large cortical infarct. This has become the most reproducible and suitable model for approximating the pathology and symptoms of human stroke [50,51]. Although pMCAO with intraluminal thread technique is a widely used animal model of stroke, it has an important drawback: insertion of the thread leads to obstruction of the hypothalamic artery and thus to hyperthermia, which in turn increases infarct volume, worsens functional outcome [52,53] and confounds drug evaluation [54]. In addition, this model shows variability in infarct volumes and higher mortality rates [55], limiting long-term survival studies. The Tamura model permits assessment of neuroprotection over a longer period of time. Although reliable and replicable, this model does have limitations in approximating some aspects of the stroke injury. In particular, it produces only a cortical infarct, so it is not a good model of sub-cortical ischemic stroke.

In stroke patients, sensorimotor deficits can persist for long periods. The sensory neglect test using the adhesive removal task is used in rats to evaluate long-term sensory-motor effects of brain injury [56] and is sensitive in assessing minute asymmetric deficits and long-lasting recovery in models of cortical damage induced by distal MCAO [57]. In the present study we found that progesterone treatment ameliorated sensory neglect at 3 and 6 weeks after pMCAO but by 8 weeks enough spontaneous recovery had occurred to mask a treatment

effect. The rats' grip strength and ability to remain on the rotarod, impaired by the injury, were also significantly improved in the progesterone-treated group at 3 and 8 weeks post-pMCAO.

An overwhelming majority of older stroke patients have cognitive deficits compared to younger counterparts [58]. The MWM has been widely used in rat MCAO models as a cognitive task to test acquisition [59–62] and long-term retention [62]. We used MWM learning acquisition and a probe trial to assess cognitive and memory deficits after pMCAO. The pMCAO group showed long-term spatial learning and memory impairment compared to shams, a finding consistent with earlier studies [62–65]. In contrast, short-duration progesterone treatment showed progressive reduction in the latency to find the platform and increased the time spent in the platform quadrant (a sign of improved memory) at 8 weeks after the injury.

Gait changes are considered reliable and quantitative indices of stroke severity and efficacy of rehabilitative therapies in humans. Stroke animals exhibit subtle but persistent gait impairments similar to impairments in patients with hemiplegic stroke such as decreased cadence and base of support [66–68]. Hetze *et al.* also showed decreases in maximum stride length, contact area and swing speed in the impaired hind limb following transient MCAO in mice [69]. Hemiparetic patients show similar deficits in swing initiation and forward propulsion after stroke [70]. Our long-term analysis of gait parameters together with motor, sensory and memory tests could be a reliable predictor of the long-term effects of potential therapeutic agents given only in the acute stages of the stroke. We found significant reduction in stride length, paw print and swing speed in the pMCAO-only group compared to shams up to 8 weeks after the injury, and progesterone treatment improved performance on all of these gait outcome measures.

Although cerebral infarct has been used frequently to assess potential neuroprotective agents, it does not always correlate well with gains in functional improvement. For example, the σ -1 selective agonist SA4503 was found to improve functional recovery one month post-stroke, but did not affect stroke volume [71]. We and others have previously reported that progesterone treatment reduces infarct size in stroke models at earlier time points [7–12,49,72–79]. In the present study we observed that 3-h delayed progesterone treatment after pMCAO produced a significant reduction (62.56%) in the volume of infarct (% contralateral hemisphere) at 8 weeks post-injury compared to the vehicle-only group. We earlier reported that 3-h delayed progesterone treatment produced a similar reduction (59.29%) at 3 weeks post-stroke [8].

After brain injury, including stroke, GFAP expression increases in astrocytes, and activated astrocytes release inflammatory cytokines, upregulate iNOS, and disrupt functional recovery as a result of this inflammatory cascade [80–82]. A large body of literature demonstrates progesterone to be a potent anti-inflammatory agent [83] that can modulate microglial activity after brain injury [84] in both *in vivo* and *in vitro* models of ischemia [13]. In the present study we found that progesterone markedly decreased gliosis (GFAP and Iba1 immunostaining) which is reflective of insidious inflammatory processes that can persist

long after an initial brain injury. Our results are in agreement with previous studies at earlier time points showing sustained, progesterone-induced reduction in GFAP expression [9].

Despite abundant positive pre-clinical research in multiple laboratories worldwide, the failure of both phase III TBI clinical trials [85,86] may lead some to question the translational potential of progesterone treatment for stroke. However, while there are some similarities, stroke and TBI are different disorders, with different consequences and mechanisms. In addition, for their primary outcome measures, the clinical trials employed subjective patient and rater quality-of-life surveys rather than quantitative assessments of functional loss or gain following stroke and treatment (see [28] for an extended discussion of the issues). Such subjective measures cannot be used in animal studies because animals cannot be asked how they feel about the quality of their lives and because subjective reports they do not register the subtle changes in both short- and long-term evaluation of functional recovery [87,88]. In summary, our current study demonstrates that, when given at the right doses and with the right duration of treatment, even with a relatively short course of treatment, progesterone can confer substantial long-term functional and histologic protection in a model of pMCAO employing quantitative assessment measures over time. These findings, along with other recent studies in multiple models of stroke, lend further support to the potential for the clinical investigation of progesterone in stroke predicated on the use of appropriate quantitative, histological, and behavioral measures.

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Research Highlights

- Progesterone was evaluated for neuroprotection in older rats with permanent stroke.
- Progesterone treatment improved long-term motor, sensory and learning function.
- Progesterone-treated animals showed attenuation of infarct volume.
- Progesterone treatment decreased glial fibrillary acidic protein expression.

Figure 1.

Effect of delayed treatment with progesterone on permanent middle cerebral artery occlusion-induced deficits in grip strength (**A**), rotarod (**B**), sensory neglect as measured by mean latency to remove stickers from contralateral forepaws (**C**), spatial learning (**D,E**), and a memory task assessed by percent time spent in the platform quadrant during a Morris water maze probe trial (**F**). Values are expressed as mean $\pm$ SEM. Sham (n= 9), pMCAO-vehicle (n=10), and pMCAO-PROG (n=10). Significant difference * p <0.05 compared to sham and # p <0.05 to vehicle.

Figure 2. Effect of delayed progesterone treatment on permanent middle cerebral artery occlusion-induced gait impairments
A. Stride length; **B.** Print area; **C.** Swing speed. Values are expressed as mean $\pm$ SE. Sham (n= 9), pMCAO-vehicle (n=10), and pMCAO-PROG (n=10). Significant difference * p <0.05 compared to sham and # p <0.05 compared to vehicle.

Figure 3. Effects of delayed progesterone treatment on infarct volume

A. Cresyl violet-stained coronal sections from representative animals given either vehicle or progesterone. Infarcts are shown as pale (unstained) regions involving the cortex. **B.** Delayed progesterone treatment reduces infarct volume at 56 d post-permanent middle cerebral artery occlusion (pMCAO). Compared to vehicle alone, progesterone significantly reduced infarct volumes (% of contralateral hemisphere). pMCAO+vehicle (n=8), pMCAO+PROG (n=8). The data are represented as mean $\pm$ SE; * p <0.01 = significant difference compared to pMCAO+Vehicle.

Figure 4. Astrogliosis

Immunohistochemical staining for glial fibrillary acidic protein (GFAP) in the peri-infarct area at 8 weeks after permanent middle cerebral artery occlusion. The DAPI in the brain is labeled as blue (**A**, **E** and **I**) and GFAP astrocytes were labeled as green. Immunostaining images show higher intensity of GFAP in the vehicle group (**F**) compared to the control (**B**) group. Treatment with progesterone reduced the intensity of GFAP (**J**). The scale bar represents 10 μ m. The lower panel bar graph shows the quantification of GFAP intensity at 8 weeks post injury. Data expressed as mean $\pm$ SE (n = 6/group).

Significant difference * $p < 0.05$ compared to sham and # $p < 0.05$ compared to vehicle.

Figure 5. Microgliosis

Immunohistochemical staining for Iba1 in the peri-infarct area at 8 weeks post-pMCAO.

Immunostaining images show higher intensity of Iba1 in the vehicle group (D) compared to control (A) group. Treatment with progesterone reduced the intensity of Iba1 (G). The scale bar represents 10 μ m. Lower panel bar graph shows the quantification of Iba1 intensity at 8 weeks post injury. Data expressed as mean $\pm$ SE (n= 6/group).

Significant difference * p <0.05 compared to sham and # p <0.05 compared to vehicle.