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Research report

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

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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.

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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 measres 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.

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1. 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 preclinical 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) antagonist NBQX [5], N-type calcium channel antagonist Ziconotide (SNX-

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-b-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, Zicontide; STAIR, Stroke Treatment Academic Industry Roundtable; TBI, traumatic brain injury.

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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 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. 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-b-cyclodextrin (HBC; Sigma-Aldrich, St. Louis, MO, USA) or progesterone (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] 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 interiorly 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

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