

Effects of progesterone on brain infarct size and locomotor activity in transient focal cerebral ischemic male rats

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Abstract

The aim of present study was to evaluate the effectiveness of progesterone (PROG) on volume infarct size, locomotor and emotional activity in cerebral ischemic rats. Thirty two male wistar rats were used in this study. Animals were divided into three groups: sham, ischemia+PROG and ischemia+vehicle. In order to induce ischemia, middle cerebral artery was occluded for 30 minutes using middle cerebral artery occlusion (MCAO) model. Then animals were tested in Open field, Hole board and Longa neurological tests 24 h later. Brain slices were stained with Tetrazolin Hydrochloride and analyzed using the NIH image analyzer software. Statistical analysis showed a significant reduction in infarct volume ($p < 0.01$), and significant improvement in locomotor activity in ischemia+PROG compared to the ischemia+vehicle group. There was no significant difference in anxiety indices in the PROG – treated group compared to vehicle. Improvement in infarct size and locomotion indices indicate that PROG may act as a neuroprotective agent in the animal model of ischemia.

Keywords: Brain, Ischemia, Progesterone, Rats, Locomotor activity

Introduction

Stroke remains one of the leading causes of death and disability worldwide, and has significant clinical and socioeconomic impact. Elkins and Johnson have projected that fatalities due to stroke will increase exponentially during the next 30 years in the United States [1].

Ischemic stroke accounts for approximately 80% of all stroke incidences, and results from a thrombotic or embolic occlusion of a major cerebral artery, often middle cerebral artery or its branches [2]. Sudden loss of blood circulation to an area of the brain results in a corresponding loss of neurological function.

After cerebral ischemic injury, two areas are formed: the core part, consisting of dead cells and the penumbra around it. The main goal of treatment in stroke victims is to rescue cells in the penumbra area, where necrosis has not occurred yet. This will provide a time window for initiation of the therapy, which necessitates the use of drugs that interrupt the process of necrosis [3].

Currently, drug therapy options for stroke are limited, and many promising drugs have failed in human clinical trials due to intolerable side effects or therapeutic limitations. These failures are to some extent attributable to the lack of appropriate pre-clinical models.

Human studies have shown that the incidence of stroke in premenopausal women is lower than that in the same age men, and it is well known that the incidence of cerebrovascular accidents rapidly increases in women after menopause [4]. Indirect evidences indicating female sex hormone as neuroprotective agents first arose from studies in intact animals. Studies in rats and mice found that young adult female animals having smaller infarct volume as compared to males following MCAO [4, 5]. Female rats have also been reported to have greater survival rates as compared to males following traumatic brain injury [6, 7, 8]. Administration of estrogen significantly decreased infarct size by approximately 50% within the first 4 hours following

MCAO in male rats [9]. Also PROG treatment was able to reduce the consequences of transient cerebral ischemia [10] and normalize the levels of proliferation and cell death in the hippocampus of traumatic brain [11, 12]. Surprisingly Murphy and coworkers reported that PROG exacerbates striatal stroke injuries in ovariectomized young female rats. Regarding clinical importance of neuroprotective agents and inconsistency in the existing literatures, this study was designed to evaluate the effects of PROG on infarct brain volume, some of the neurological and emotional characteristics after MCAO in male rats.

Material & methods

Experimental procedures

Male Wistar rats weighing 180-230 g were obtained from Guilan University of Medical sciences animal center (Rasht, Iran). Rats were maintained at temperature between $22 \pm 2^{\circ}\text{C}$, with a 12 h light-dark cycle. Experiments were conducted in accordance with the Animal Ethics Committee of the Guilan University of Medical Sciences.

Surgical procedures & treatment

In this study we used the MCAO technique for inducing middle cerebral artery occlusion [13, 14]. Animals were anesthetized with 400 mg/kg chloral hydrate (Merck chemical company), i.p. Then nylon tread 40 (Ethicon -UK) was prepared by rounding its tip by heating and coating with poly-L-lysine (Sigma). The right carotid region was exposed and nylon tread was introduced through the right external carotid artery into the internal carotid artery. The nylon tread was left in place for 30 minutes and then pulled out. Wound area was sutured and animals were kept in a heating blanket until recovery. Two hours after surgery animals received s.c injection of PROG (Tolid Daro Company, Tehran, Iran) (8 mg/Kg) or sesame oil.

Behavioral tests

Behavioral tests were performed 24 hours after surgery by recording the behavior of the animals and scoring by a second person unaware of the treatments. Neurological deficits in the vehicle and PROG treated groups were measured according to the method of Longa [13] scores used in this study were as follows:

- Score 0: no apparent neurological deficits
- Score 1: failure to extend right four paw fully
- Score 2: circling to the right
- Score 3: falling to the right
- Score 4: no spontaneous walking with a depressed level of consciousness

Open field

Animals were placed in an open field apparatus which consisted of a circular container with 80 cm diameter and 40 cm height. The bottom of the cylinder was divided into 60cm and 30cm diameter circles. A 100w lamp illuminated the field, and a camera was placed on the top of the container and the behavior of the animal was recorded for 5 minutes. In this experiments, the number of peripheral ambulation, central ambulation, first latency to move, latency to center, peripheral rearing, central rearing, grooming and exploration were recorded by camera. This test was used to measure the movement, excitability, emotionality and activity of autonomic system as well.

Hole board

Hole board apparatus was used to measure the first latency and exploration of the animals. For this experiment animal was placed individually, in a 34 × 24cm wooden box with 10 holes on the top of the box. For exploration animals had to stand on their hind limb. Animals' behavior was recorded for 3 minutes.

Histology

At the end of the experiments animals were decapitated, and the brains were removed and placed in a brain matrix (Harvard Apparatus; Holliston, MA, USA), and 2mm coronal slices were cut and incubated for 30 min at 37 °C in a 2% solution of 2, 3, 5- triphenyl tetrazolium chloride followed by fixation in 10% formalin. Ischemic lesions turned white while healthy tissues were stained red. Brain slices were photographed using a Cannon digital camera and analyzed with NIH Image analyzer program. The total ischemic lesion volume was calculated as the sum of the volume of the ischemic lesions across the slices in the cortex and striatum separately.

2.6. Statistic

Statistical analysis was conducted using the SPSS software system (ver. 19). Mann –Whitney U test and student's t test were used for analyzing non-parametric and parametric data respectively. Level of significance set at $p < 0.05$.

Results

2.1 Effects of MCAO on behavioral parameters in open field

As Fig 1 shows there was a significant difference in all of the locomotor and emotional parameters in open field, including: peripheral ambulation, central ambulation, latency to move ($P < 0.01$), latency to center, peripheral rearing, central rearing, grooming and exploration ($p < 0.001$) in ischemic compared to sham operated group. Behavioral and locomotor disturbances following MCAO surgery indicate successful modeling of ischemia.

see Fig. 1

2.2 Effects of PROG on infarct volume

There was a significant increase in percentage of brain infarct volume in MCAO vs. sham operated

groups. Progesterone administration significantly reduced the total infarct volumes in the cortex after MCAO compared with vehicle-treated rats (3.62 ± 1.04 vs 21.75 ± 3.78 ($p < 0.01$)).

see Fig 2.

see Fig 3.

2.3 Effects of PROG on Longa test

Animals in the PROG treated group on average scored about 1 in Longa test, compared to about 3 in the vehicle treated group. The difference between the two groups was significant $P < 0.01$.

2.4 Effects of PROG on locomotor and emotional parameters in open field

There was significant improvement in grooming, ambulation peripheral and rearing peripheral ($P < 0.01$) in the PROG -treated compared with the vehicle treated group.

No significant difference was observed between PROG treated vs. vehicle treated group in ambulation central and rearing central.

2.5 Effects of PROG on locomotor parameters in hole board

There was no significant difference in exploration and first latency to move significant between the PROG group compared to the vehicle treated group in the hole board experiment.

Discussion

The objective of this study was to evaluate the effects of PROG using different neurological tests in MCAO model. The Middle cerebral artery is the specific occlusion site in our model, which is the most commonly affected vessel in both embolic and thrombotic strokes in humans too [15].

The results of our study showed that PROG (8mg/kg) was able to partially protect the dying

neurons following focal cerebral ischemia . This dose of PROG, which is the maximal effective dose, was selected according to the previous studies in rat [12, 10] and mice [16] .

This finding is in agreement with the results from other laboratories showing significant reduction in cortical infarct volume and better neurologic outcomes after systemic administration of PROG (4mg/kg) either prior to MCAO or 2h after reperfusion [17,18].

It is well known that MCAO results in behavioral, neurochemical and histological abnormalities in rat brain and production of free radicals [19] . Muscle weakness or motor impairment is a common complaint after stroke in humans. It is important for the pathophysiological studies and development of drug therapies for stroke to use appropriate behavioral testing in conjunction with histological measurement [20, 21]. In this study, we used three different behavioral tests: Longa's neurological measurement, open field and hole board in order to evaluate behavioral and neurological effectiveness [13]. Consistent with other researcher's observations, we found that behavioral deficits caused by MCAO were significantly improved by PROG treatment, 24h after the surgery [10, 18]. Also our results are in agreement with studies which used other neurological tests such as accelerating and grip strength [16, 22]. In our study the degree of improvement was significant compare to vehicle treated groups (approximately returning to normal function).

According to the open field data, after the treatment, locomotor indices such as ambulation, rearing and exploration improved more than the emotional indicators such as latency to the center part of area. Moving to the center part of the open field and exploratory activity in that part are considered strong indices of decreased anxiety levels [23, 24]. It is possible that the lack of improvement in some of the emotional indices might be due to the time frame that the tests were conducted (24 hours post treatment). Studies with different time intervals are needed to investigate the effects of time on

recovery in anxiety state of the animals. However, we cannot exclude the possibility that ischemia affects on different brain regions involving in the emotionality, and the dose of 8mg/Kg of progesterone failed to recover those abnormalities. Moreover, histological experiment revealed more improvement in infarct volume in the cortex of PROG- treated group rather than striatum.

Although molecular mechanisms of ischemia and progesterone on locomotion was not the goal of this study, there are good evidences in the literatures that ischemia reperfusion injury causes a significant increase in reactive oxygen species, lipid peroxidation, nitrite concentration [25, 26, 27] and cytokines [28]. It has been proposed that PROG acts via genomic and non genomic mechanisms. Progesterone exerts its action primarily through the intracellular membrane-bound PROG receptor by inhibiting NMDAR-Ca²⁺ influx and activation of PR-mediated Src-ERK signaling pathways [29]. In rat model of TBI, acute PROG treatment helped to reduce inflammatory factors such as COX-2 and IL-6 at various time points after the injury. Since the stroke model also induces edema[30], it is likely that post-stroke administration of PROG would have similar benefits .

Taken together, our findings reveal that progesterone ameliorates infarct size, and improve locomotor activity in the MCAO model.

Conclusions

In conclusion, PROG treatment significantly reduced infarct volume and improved functional deficits in a clinically relevant model of stroke.

In a clinical point of view, progesterone might be a pre-clinical promise as a potential therapeutic strategy for stroke patients. Further investigations are needed to evaluate time–response mechanisms of action of PROG in an experimental permanent stroke model.

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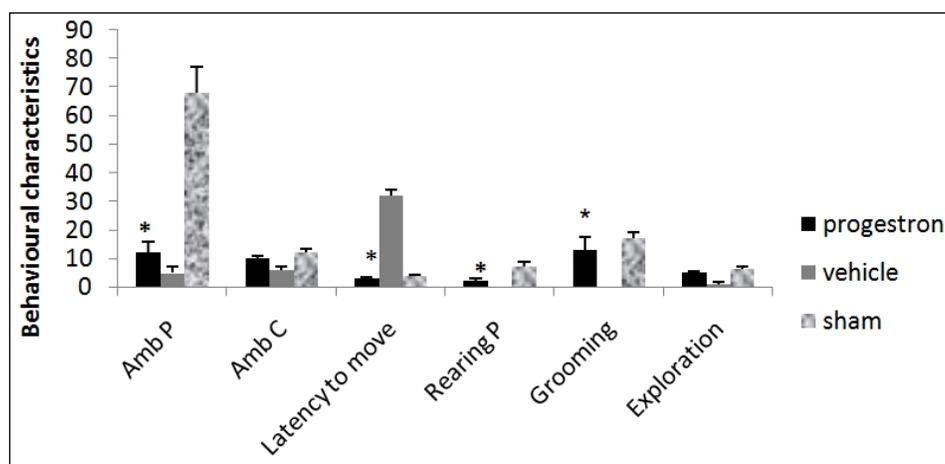


Fig 1. Behavioral characteristics of treated groups in open field. PROG causes significant reduction in locomotor indices like peripheral ambulation (Amb p), latency to move, peripheral rearing (rearing p), and grooming



Fig. 2

Fig. 2: A=up, B=down.

Effect of PROG on infarct size. Pictures show representative examples of TTC-stained brain sections from Ischemic+vehicle (up) and Ischemic+PROG (down) treated groups. The unstained white area represents infarcted tissue. The infarcted area in PROG-treated is substantially reduced

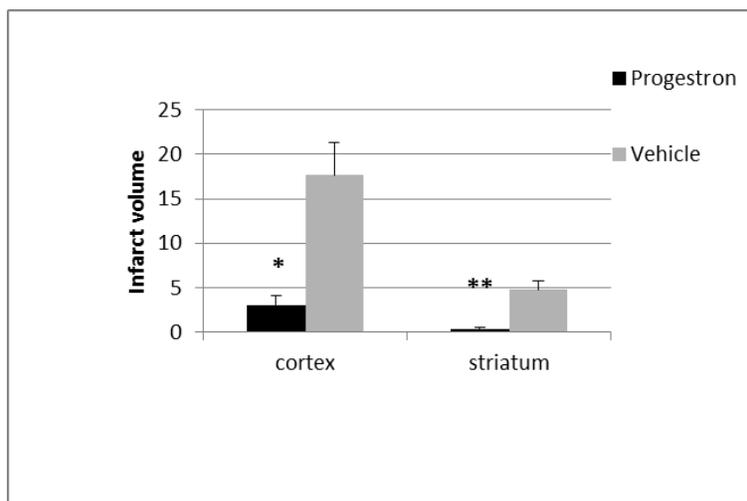


Fig. 3

Fig. 3. There was a significant increase in percentage of brain infarct volume in MCAO vs. sham operated groups. PROG treatment causes significant reduction in infarct volume in cortex $P < 0.001$ and striatum $P < 0.01$ compared to vehicle treated group.