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Progesterone improves functional outcomes after transient focal cerebral ischemia in both aged male and female rats

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ABSTRACT

Progesterone hormone (P4) is a promising agent against strokes because post-ischemic administration of P4 exerts neuroprotective effects in several young and aged animal models of stroke. However, in contrast to a majority of the studies using male animals, female animals remain underrepresented. In addition, we do not know whether the same administration way of P4 is effective in both male and female animals because there are gender different responses to steroid hormones and stroke. In this study, we thus evaluated long-term histological and functional outcomes in the same treatment with P4 in both 18-month old male and age-matched female rats subjected to transient middle cerebral artery occlusion (MCAO). MCAO aged male and female rats were given a subcutaneous injection of P4 (4 mg/kg) 6 h after MCAO followed by once daily for successive 7 days. The post-ischemic administration of P4 significantly improved the impairments of spatial working memory and motor coordination 28–29 days after MCAO in both aged male and age-matched female rats. However, the P4 administration slightly but not significantly reduced infarct sizes 30 days after MCAO in aged female rats, in contrast to significant better histological outcome in P4-treated aged males. On the other hand, these histological and behavioral analyses showed no adverse effects of P4 in aged rats of both sexes. Collectively, our study provides preclinical evidence to prompt further preclinical studies for post-stroke treatment with P4 and the translation of its clinical trials in old stroke patients of both sexes.

1. Introduction

Strokes are a major serious health issue in a large number of countries, while available therapeutic approaches against strokes are still limited. Therefore, there is an urgent need to develop safer and more effective therapeutic agents against strokes. Progesterone hormone (P4) is spotlighted as a promising agent against strokes because there is a large number of evidence demonstrating that post-ischemic administration of P4 exerts neuroprotective effects in several young and aged animal models of stroke (Cai et al., 2012; Chen et al., 1999; Gibson et al., 2011; Wali et al., 2014). However, a majority of studies on post-stroke treatment with P4 has been done in male animals. Female animals remain underrepresented in the experimental research, and the assessment of long-term outcomes in P4-treated female animals is lacking (Gibson et al., 2011). Especially, it is beneficial to evaluate effects of P4 on strokes in postmenopausal model-aged female animals because menopause is associated with multiple risk factors of cardiovascular diseases (Lisabeth and Bushnell, 2012). Moreover, there are different hormone backgrounds in the brain and hormone-independent ischemic neuronal cell death pathways between male and female animals (Guerra-Araiza et al., 2002; Lang and McCullough, 2008; Liu et al., 2011; Liu et al., 2009; MacLusky et al., 1985). Therefore, the same administration way of P4 may not bring about reliable neuroprotection against strokes in both male and female animals. In this study we thus evaluated long-term histological and functional outcomes in the same treatment with P4 against experimental strokes both in 18-month old male and age-matched female rats.

2. Materials and methods

2.1. Animals and housing conditions

Aged male (698.5 ± 45.2 g) and female (360.0 ± 33.0 g) Sprague-Dawley rats (17–19-month-old) were purchased from Chubu Kagaku Shizai (Nagoya, Japan) and housed for > 2 months in a light-controlled room under a 12-h light/dark cycle starting at 9:00 AM, and maintained

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at a temperature of 25 °C. Animals had free access to food and water, and were handled at least six times over one week before surgery. Rats that had surgery were given agar chow in addition to solid chow everyday. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Nagoya University and carried out in accordance with the guidelines established by the Nagoya University.

2.2. Preparation of the transient focal cerebral ischemia model

Transient focal cerebral ischemia was induced by occluding left middle cerebral artery (MCAO) and bilateral common carotid arteries (CCAO). MCAO and CCAO were conducted as described previously (Tanaka et al., 2018). Core body temperatures were monitored and maintained at 36.5 ± 1 °C using a warm pad during surgery. A micro-clip (TS-93026, Unique Medical, Tokyo) was applied to the left MCA trunk at the inferior border of the olfactory tract. The bilateral CCAs were occluded by micro-clips (KN-353, Natsume, Tokyo) within 5 min of MCAO. The interruptions of the flows of the left MCA and the bilateral CCAs were carefully confirmed visually following application of the clips. After 45 min of the three vessel occlusion, the micro-clips were removed from the left MCA and then the bilateral CCAs. The reperfusion of each artery was carefully confirmed visually. Sham-operated rats were treated identically, except that the left MCA and bilateral CCAs were not occluded.

2.3. Animal grouping and drug administration

P4 (Sigma) was dissolved in 30% 2-hydroxypropyl-β- cyclodextrin (HBC, Wako; vehicle) in saline. For a dose-effect experiment, a total of 20 aged male rats were randomly given a combined intraperitoneal and subcutaneous injection of 4 (n = 6) or 8 (n = 6) mg/kg P4 or its vehicle (n = 8) 6 h after MCAO, followed by subcutaneous injections 16 h later and then once daily for the next 6 days. The last two doses were halved over the last two days. To evaluate long-term outcomes in the treatment with P4, a total of 27 aged male or 22 aged female rats were randomly assigned to one of three groups: sham-operated (n = 9 in males or 6 in females), vehicle-treated MCAO (n = 9 in males or 8 in females), or P4-treated MCAO rats (n = 9 in males or 8 in females). The vehicle- or P4-treated MCAO rats were given a subcutaneous injection of P4 (4 mg/kg) or its vehicle 6 h after MCAO and then once daily for the next 7 days. The last two doses were halved over the last two days (Fig. 1A). Drugs were prepared freshly on the day of the experiment.

2.4. Cresyl violet (CV) staining and infarct size measurements

Rats were deeply anesthetized with a combination anesthetic agent (i.p.) containing 0.15 mg/kg medetomidine, 2 mg/kg midazolam, and 2.5 mg/kg butorphanol, and transcardially perfused with Lactated Ringer's solution (Otsuka Pharmaceutical) followed by 4% paraformaldehyde in PBS 8 or 30 days after MCAO. The brains were removed, post-fixed in 4% paraformaldehyde in PBS, and then cut at 8 mm from the olfactory bulb on a rat brain slicer (Muromachi kikai). Both the anterior and posterior brains were processed for paraffin embedding. One or four-series of coronal sections (5-μm-thick) were cut every 300 μm from the cut surface of the anterior brains using REM-710 (Yamato Koki Industrial Co., Ltd., Japan). The 6th and 10th sections of each anterior brain were used for the histological analysis. The deparaffinization of specimens was performed by washing two or three times with fresh xylene (Wako) for 3 min each time followed by two washes with graded ethanol for 3 min.

For CV staining, the sections were stained with 0.1% cresyl violet (Muto pure chemicals, Tokyo) for 1 min at room temperature. The sections were quickly rinsed in distilled water adding 2 drops of acetic acid and then washed in 90% ethanol. They were subsequently dehydrated three times with absolute ethanol for 3 min and then cleared twice in xylene. Digital images were collected on BX-Z700 (Keyence). Unstained or irregularly stained areas of the brain sections were defined as infarcted areas, and measurements were performed by manually outlining the margins of these areas using the image analysis software GIMP 2. Infarct size was calculated as described previously (Swanson et al., 1990). The histological analysis was conducted by investigators blinded to the treatment or vehicle groups.

2.5. Behavioral analysis

Fig. 1B shows a timeline of the behavioral tests after transient MCAO (Fig. 1B). Animals were habituated in the experimental room for at least 1 h prior to the start of the behavioral tests. The tests were performed between 1:00 PM and 6:00 PM. The behavioral analysis was conducted by investigators blinded to the treatment or vehicle groups.

2.5.1. Y maze test

Spontaneous alternation behavior in the Y maze was used to evaluate the spatial working memory and exploratory behavior of rodents on the 28th day after MCAO (Jin et al., 2010; Yabuki and Fukunaga, 2013). The Y-maze (Brain science idea) had three arms with a 120°

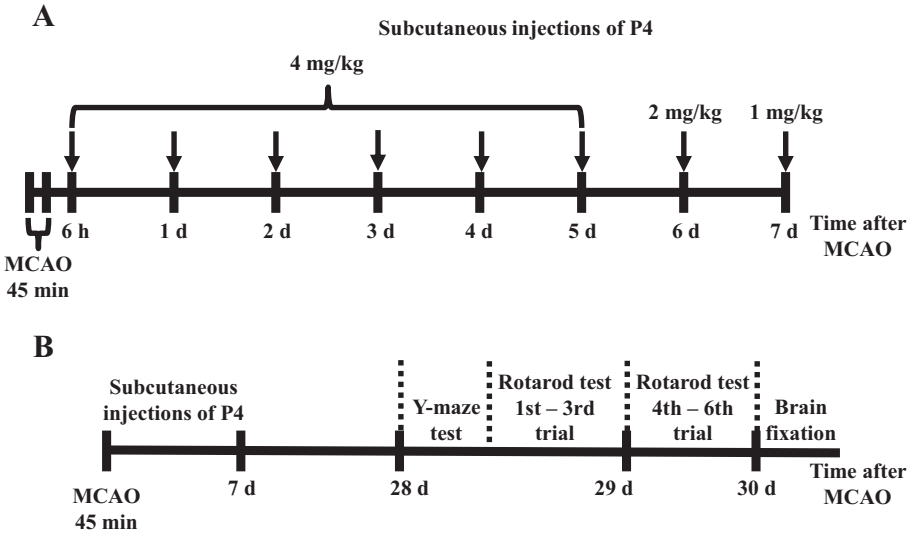

Fig. 1. Schematic experimental design to evaluate long-term outcomes in P4-treated MCAO rats. (A) A timeline of subcutaneous injections of P4 or its vehicle after MCAO. (B) A timeline of the behavioral tests and brain fixation for CV staining.