Combination treatment with progesterone and rehabilitation training further promotes behavioral recovery after acute ischemic stroke in mice

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Abstract

Purpose: The present study was aimed to investigate whether combination treatment with progesterone and rehabilitation training would be more effective than monotherapy after cerebral ischemia.

Methods: C57BL/6 mice were subjected to focal ischemia by photothrombosis and were treated with progesterone (2 mg/kg), rehabilitation training or their combination. 2,3,5-triphenyltetrazolium chloride (TTC) staining and Nissl staining were used to measure infarct size at day 3 and day 7 after surgery, and rotarod test and grip strength test were conducted to evaluate behavioral outcomes.

Results: TTC staining indicated that progesterone, rehabilitation training and their combination produced a different degree of reduction in infarct volume compared with vehicle control at day 3 after ischemia (progesterone: 16.70 ± 0.93 mm³, p < 0.01, rehabilitation training: 22.19 ± 0.93 mm³, p < 0.05, progesterone + rehabilitation training: 14.76 ± 0.92 mm³, p < 0.01, vehicle control: 28.73 ± 1.05 mm³). Nissl staining revealed that prolonged treatment of progesterone, rehabilitation training and their combination led to a significant decrease in infarct volume at day 7 after ischemia (progesterone: 18.64 ± 1.83 mm³, p < 0.01, rehabilitation training: 25.07 ± 1.70 mm³, p < 0.05, progesterone + rehabilitation training: 17.09 ± 0.92 mm³, p < 0.01, vehicle control: 30.31 ± 1.36 mm³). No accumulative effect in the reduction of infarct volume was observed in combination therapy at both day 3 and day 7 after ischemia. However, combination therapy significantly improved behavioral performances in the first week after photothrombosis. Combination treatment significantly enhanced rotarod performance and forelimb grip strength at all time points within 7 days after ischemia compared with rehabilitation alone, and significantly improved rotarod performance and forelimb grip strength from day 2 after ischemia compared with progesterone alone.

Conclusion: Our results suggested that combination treatment with progesterone and rehabilitation training had no additive effect in reducing infarct volume, but combination therapy exhibited enhanced efficacy in promoting functional recovery after ischemic stroke, suggesting progesterone and rehabilitation training may exert their effects via different mechanisms.

Keywords: Progesterone, rehabilitation training, ischemic stroke, infarct, functional recovery
Abbreviations

ANOVA Analysis of variance
PBS Phosphate-buffered saline
RB Rose bengal
SEM Standard error of the mean
TTC 2,3,5-Triphenyltetrazolium chloride

1. Introduction

Stroke is one of the main causes of death and disability. Insufficient supply of oxygen and glucose during stroke triggers a complex cascade of events with multisystem involvement, including disruption of the blood brain barrier, glial cell activation, inflammation, necrotic and apoptotic neuronal cells (Danton & Dietrich, 2003; Deb et al., 2010) and disruption of neural circuits (Wieloch & Nikolich, 2006). Previous studies suggested that therapeutic interventions targeting one aspect of the pathological cascade displayed very limited effect in clinical trials (Cheng et al., 2004; Ginsberg, 2008). Thus, it is a recommended strategy to use a pleiotropic drug or combination therapy to block or reduce a cascade of pathological events after ischemia (Zhang et al., 2012a). The present study was aimed to investigate whether the combination of two promising therapies for stroke treatment, progesterone and rehabilitation training, would have better efficacy in reducing infarct volume and brain functional deficits in a photothrombotic stroke model.

Progesterone is a pleiotropic steroid hormone. It is mainly synthesized by gonads and placenta. Studies have indicated that progesterone can also be synthesized by neurons and glial cells in the brains of both males and females (Baulieu & Robel, 1990; Schumacher et al., 2001). However, circulating levels of progesterone in age-matched men is much lower than premenopausal women. Evidences have indicated that progesterone has neuroprotective effect in different lesion models, including traumatic brain injury (O’Connor et al., 2007; Shear et al., 2002; Wali et al., 2011), cerebral ischemia (Gibson et al., 2011; Gibson & Murphy, 2004; Jiang et al., 1996; Kumon et al., 2000) and spinal cord injury (De Nicola et al., 2006; Thomas et al., 1999). Epidemiological studies have shown that premenopausal women have a lower risk of stroke compared with males of the same age (Turtzo & McCullough, 2008). The incidence of stroke increases substantially after menopause (Prencipe et al., 1997; Wenger et al., 1993), correlating to a decline in circulating steroid hormones such as progesterone and estrogen. These studies suggest that progesterone and estrogen may provide endogenous protection against stroke. The neuroprotective efficacy of progesterone in promoting functional and morphological recovery has been confirmed in both permanent and transient stroke models (Chen et al., 1999; Ishrat et al., 2009; Jiang et al., 1996; Sayeed et al., 2007). Despite beneficial effects of progesterone in ischemic stroke, the detailed mechanism remains to be elucidated for progesterone to enter clinical practice.

Rehabilitation training is a suggested intervention for stroke survivors and has been proved to be beneficial to functional recovery in both animal experiments and clinical practice (Brown & Schultz, 2010; Joo et al., 2012; Teasell et al., 2005). Constraint-induced therapy improved the function of affected upper extremity post stroke in clinical studies (McIntyre et al., 2012; Siebers et al., 2010). Rehabilitation training after stroke may exert neuroprotective effect by reducing infarct volume, increasing synaptic plasticity and improving functional recovery (Adkins et al., 2008; Matsuda et al., 2011). The earlier rehabilitation training is performed, the better efficacy rehabilitation training will exert (Bland et al., 2000; Yang et al., 2003; Zhang et al., 2012b). Effect of rehabilitation training may be associated with factors such as task specificity, training intensity, frequency of training and complexity (Fraser et al., 2002; Hornby et al., 2011). However, rehabilitation training alone has limited effects in improving functional recovery, and it is often used together with other therapeutic interventions to increase therapeutic efficacy in stroke treatment. Progesterone and rehabilitation training have different therapeutic profiles that might target different pathological aspects of stroke. Progesterone may exert neuroprotection by targeting many aspects of the pathological cascade (Sayeed et al., 2009; Wang et al., 2009; Wang et al., 2011), and rehabilitation training may be mostly related to increasing reorganization of cortical maps (Kopp et al., 1999; Traversa et al., 1997). In this study, we evaluated whether combination treatment with progesterone and rehabilitation training would have better therapeutic efficacy in ischemic stroke. We assessed the effects of progesterone and rehabilitation training by using a combination of histological and behavioral assays in a photothrombotic stroke model in mice.
2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice, weighing 22 to 25 g, were used for the experiments. Animals were housed on a 12:12-h light/dark cycle and environmental temperatures were maintained at 18–22 °C. Food and water were freely available. All animals were handled and cared for in accordance with the ‘Guide of the Care and Use of Laboratory Animals’ approved by the ethic Committee of Experimental Animals of Lanzhou University.

2.2. Ischemic model

Cerebral focal ischemia was induced by pho-thrombosis (Watson et al., 1985; Zhang et al., 2005). C57BL/6 mice were anesthetized intraperitoneally with ketamine (100 mg/kg body weight) and xylazine (15 mg/kg body weight). Rectal temperature was maintained at 37 °C using a heated blanket with feedback control (JR-1/2, Taimeng, China). The scalp was incised from midline and the skull was exposed. To immobilize the head, the skull was glued to a stainless steel plate, which was screwed down to two lateral bars on a metal base. The sensorimotor region of cortex (2.2 mm lateral; 0.8 mm posterior of bregma) was chosen and a ~1.0 × 1.0 mm region of skull was thinned using a high-speed dental drill. Mice were injected with 0.6% rose bengal (RB) in phosphate-buffered saline (PBS) (24 mg/kg) via the tail vein. The cortical microvessels of the thinned region were illuminated with a beam of green light for 4 min 15 sec. The scalp was sutured after stroke induction. Mice were monitored until fully awake and then were returned to their home cages. Sham surgery controls were treated in an identical manner but without green light illumination. The mice were supplemented with 0.1 ml solution of 0.5% (w/v) glucose in PBS via the tail vein. The cortical microvessels of the thinned region were illuminated with a beam of green light for 4 min 15 sec. The scalp was sutured after stroke induction. Mice were monitored until fully awake and then were returned to their home cages. Sham surgery controls were treated in an identical manner but without green light illumination. The mice were supplemented with 0.1 ml solution of 0.5% (w/v) glucose in PBS via the tail vein.

2.3. Experimental groups and treatments

Animals were randomly divided into seven groups: (1) sham plus vehicle (n = 10); (2) sham plus progesterone (n = 8); (3) sham plus rehabilitation training (n = 10); (4) ischemia plus vehicle (n = 10); (5) ischemia plus progesterone (n = 10); (6) ischemia plus rehabilitation training (n = 10); (7) ischemia plus progesterone and rehabilitation training (n = 10). Progesterone (P-0130; Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in 22.5% 2-hydroxypropyl-β-cyclodextrin and was given at a dose of 2 mg/kg. All animals received an initial subcutaneous injection of progesterone in 22.5% 2-hydroxypropyl-β-cyclodextrin or equivalent volume/weight of vehicle (2-hydroxypropyl-β-cyclodextrin) 4h after surgery, followed by once per day until day 2 or day 6 after surgery. A person blinded to the treatment or vehicle groups conducted the measurements and evaluations.

2.4. Rehabilitation training

Mice of rehabilitation training groups and combination group were subjected to rehabilitation training, including hanging wire training, rotarod training and climb ladder training from day 1 to day 6 after surgery.

2.4.1. Hanging wire training

Hanging wire training was used to improve limb grasping ability. Mice used their forelimbs to suspend their body on an iron string (diameter: 3 mm) stretched midway between two posts (75 cm long suspended horizontally, 50 cm above ground). Mice were trained to reach one of the two ends from the middle of the wire string two times per day. The mice were forced to use injured limb to promote its recovery.

2.4.2. Accelerated rotarod training

The mice were trained on a rotarod apparatus two times per day. Rotational speed was set at 10 rpm, and then was accelerated to 20 rpm within 5 min. Mice that fell before 5 min were returned onto the rod until the training was over. The aim of the training was to improve sensorimotor coordination.

2.4.3. Climb ladder training

A 52 cm long ladder leaning against a wall was used for climb ladder training. The interval between two grades was 2 cm. The mice were trained to climb up and down two times per day. The training was to promote sensorimotor recovery of injured limb.
2.5. Behavioral tests

2.5.1. Rotarod test

The rotarod test was performed to evaluate motor coordination and balance. Before surgery, all animals were first pre-trained on the rotarod apparatus for 3 days. Mice were first placed on the rod for 3 min without rotation, and then the speed of the rotarod was accelerated from 10 rpm to 40 rpm within 5 min. Each mouse that fell was returned on the rod until the training was over. The training consisted of three sessions. Each session included three separate trials, and there was a 5 min interval. Mice were evaluated in order to select those able to walk on the rotating rod under the same conditions used in the test. After 3-day training, most animals in our studies surpassed 200 s of walking on the rotarod. Only those able to walk on the rotarod for at least 200 s were used in the experiment. The length of time walking on the rotarod was recorded in the test. Two consecutive passive rotations, without walking, but accompanying the rod were considered as a fall. The mean was used for statistical analysis.

2.5.2. Grip strength test

The forelimb grip strength test was performed with a homemade grip device attached to a highly sensitive force transducer (Xinhang, China, JZ300) that measures peak force of the forelimbs. The mouse tail was dragged backwards at constant speed when both forelimbs gripped the grasping-bar of a metal triangle stand. When mouse forelimbs loosened, the maximal force was recorded by the Biological Data Acquisition & Analysis System (Taimeng BL-420F in China). Grip strength was measured five times for each animal and the mean was recorded and used for statistical analysis. Before surgery, the mice with the forelimb grip strength surpassing 108 g were chosen to use for experiments.

2.6. Histological analysis

Mice were divided into two groups. One group were sacrificed with an overdose of urethane (0.12% w/w) at 72 h after surgery for lesion volume analysis by TTC staining. The brains were carefully removed and placed in 2% TTC (Sigma, St. Louis, MO) solution in 0.1 M PBS (pH 7.4) for 30 min at room temperature in dark and then stored in phosphate-buffered 4% paraformaldehyde for 30 min before analysis. The caudal face of each section was photographed and infarct area (unstained tissue) was measured by Image J software. The total infarct volume was calculated by summing the infarct area in each section and multiplying by the distance between sections of 1 mm thickness. The infarct volume was calculated and corrected for edema using a formula described before (Loihl et al., 1999): contralateral hemispheric volume – ipsilateral uninfarct hemispheric volume.

For Nissl staining, animals were transcardially perfused and decapitated at day 7 post-surgery. The brains were dipped in 4% paraformaldehyde for 3 days and sectioned into 30 μm coronal slices in a vibrating microtome (LEICA TV1000S). Brain sections were collected serially at 120 μm intervals and stained with cresyl violet. Infarct volume was measured using Image J software. To eliminate the contribution of post-ischemic edema to the volume of infarct, infarct volumes were corrected for swelling according to the method of Loihl et al. (Loihl et al., 1999). The resulting Nissl staining displayed dark blue color in a normal region, but light blue color in the infarct area of the brain.

2.7. Statistical analysis

All results were expressed as the mean ± standard error of the mean (SEM). All analysis of variance for repeated measures were performed with SPSS 16.0 statistical software. The behavioral tests were subjected to two-way ANOVA analysis of variance. Mean comparisons were used for Post Hoc Tests analyses of repeated ANOVAs. Cerebral ischemic volumes were analyzed by One-way ANOVA. Mean comparisons were used for Post Hoc Tests analyses of One-way ANOVA. Value of $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered statistically highly significant.

3. Results

3.1. Induction of permanent cerebral ischemia using photothermобosis

Photothrombotic lesion model was used to induce cerebral ischemia. The right sensorimotor cortex was illuminated by green light for 4 min 15 sec. After
injecting RB dye, we induced a cortical lesion with a size of $\sim 4.0 \times 5.0 \text{mm}$ by photoactivating RB in sensorimotor cortex. The lesion was located in the frontal and parietal cortex and exhibited a consistent pattern of ischemic damage, as shown in Fig. 1A. The white tissue in TTC staining was induced by photothrombosis and indicated lesion site. TTC staining can reflect mitochondrial function and is a reliable indicator of ischemic lesion for up to 3 days post-ischemia (Lin et al., 1993). Ischemia by photothrombosis in animals treated with vehicle (control) resulted in a large infarct ($28.73 \pm 1.05 \text{mm}^3$, $n=6$, Fig. 1B) at day 3 after ischemia. As assessed by Neurological scores and behavioral tests, the lesion of sensorimotor cortex resulted in significant neurological deficits. Neurological deficit scores 4 h after ischemia reached 2–3. The time walking on the rotarod by rotarod test in ischemia plus vehicle group ($150.66 \pm 9.76 \text{sec.}$, $n=10$) was significantly lower than sham control group ($244.44 \pm 6.63 \text{sec.}$, $p<0.01$, $n=10$) at first day following stroke (Fig. 2A). The forelimb grip strength assessed by grip strength test in ischemia plus vehicle group ($86.64 \pm 2.32 \text{g}$, $n=10$) decreased significantly compared with sham control ($118.11 \pm 1.52 \text{g}$, $p<0.01$, $n=10$) (Fig. 2C).

3.2. Effect of progesterone, rehabilitation training and their combination on infarct volume following ischemic stroke

To assess the effects of progesterone, rehabilitation training and their combination on infarct size, we determined the effect of subcutaneously injected progesterone on infarct volume at day 3 after ischemia. We found that the infarct volume was reduced significantly by 41.85% ($28.73 \pm 1.05 \text{mm}^3$, $n=6$, progesterone: 16.70 $\pm 0.93 \text{mm}^3$, $n=6$, $p<0.01$) after the treatment with 2 mg/kg of progesterone (Fig. 1B). We next evaluated the effect of rehabilitation training on infarct volume. Our data suggested that rehabilitation training alone decreased infarct volume significantly by 22.77% ($28.73 \pm 1.05 \text{mm}^3$, rehabilitation training: 22.19 $\pm 0.93 \text{mm}^3$, $n=6$, $p<0.05$) (Fig. 1B). The difference in the reduction of infarct volume between progesterone group and rehabilitation group was significant ($p<0.05$), suggesting progesterone had a greater neuroprotective effect than rehabilitation. We then evaluated whether progesterone and rehabilitation training administered together would reduce infarct volume further. We found that the combination of progesterone and rehabilitation training reduced infarct volume by 48.63% ($28.73 \pm 1.05 \text{mm}^3$, progesterone + rehabilitation training: 14.76 $\pm 0.92 \text{mm}^3$, $n=6$, $p<0.01$) (Fig. 1B). The reduction was significant compared with rehabilitation training alone ($p<0.05$), but was not significant compared with progesterone alone ($p=0.465$) (Fig. 1B). These data indicated that...
combination treatment with progesterone and rehabilitation training had little accumulative effect in the reduction of infarct volume.

To study the effects of progesterone, rehabilitation training and their combination on infarct size after treatment over an extended period of time, we measured infarct volume at day 7 after ischemia. We used Nissl staining to measure infarct size at day 7 after ischemia, as TTC staining doesn’t reflect infarct size after 3 days following stroke (Lin et al., 1993). The results of Nissl staining indicated that infarct area was located between Bregma anterior 1.54 mm and Bregma posterior 1.94 mm, covering right sensorimotor cortex (Fig. 3A, B). Measurements from Nissl staining sections displayed that the infarct volume in ischemia plus vehicle group is 30.31 ± 1.36 mm³ (Fig. 3C). We then assessed infarct size after the treatment of progesterone or rehabilitation training. We found that the infarct volume was significantly reduced by 38.49% (vehicle: 30.31 ± 1.36 mm³, n = 10, progesterone: 18.64 ± 1.83 mm³, n = 6, p < 0.01) after the treatment of progesterone compared with ischemia plus vehicle group, and rehabilitation training significantly reduced infarct volume by 17.28% (vehicle: 30.31 ± 1.36 mm³, rehabilitation training: 25.07 ± 1.70 mm³, n = 7, p < 0.05) (Fig. 3C). Consistent with our data of TTC staining at day 3 after ischemia, the results of Nissl staining at day 7 after ischemia also indicated that progesterone was more beneficial than rehabilitation training.
Fig. 3. Infarct volume showed by Nissl staining at 7 days after stroke. (A) Representative Nissl stained coronal section treated with vehicle at 7 days after stroke. (B) Representative coronal sections corresponding to boxed region in (A) illustrating a typical infarct from Bregma anterior 1.54 mm to Bregma posterior 1.94 mm. (C) Calculated infarct volumes at 7 days after ischemia. Progesterone, rehabilitation training and combination treatment significantly reduced infarct volume. Combination therapy significantly decreased infarct size compared to rehabilitation training alone \((n=7, p=0.001)\), but didn’t reduce infarct volume further compared to progesterone alone \((n=6, p=0.204)\).

3.3. Combined progesterone with rehabilitation training further improved sensorimotor function

We evaluated the behavioral performances after progesterone treatment, rehabilitation training and combination therapy over the course of the experiment. Rotarod test was used to evaluate neurological deficits and brain functional recovery after brain injury. Our statistical results showed that the mean scores for rotarod activity were significantly different between time points and there were significant group effects. Rotarod performance decreased slightly at the first day after surgery in sham group, but returned to and maintained to pre-surgery level from the second day (Fig. 2A). There was no significant difference between sham controls \((n=10)\), progesterone, \(n=8\); rehabilitation, \(n=10\) at different time points within 7 days post-surgery, suggesting that progesterone or rehabilitation has not significantly increased the time of walking on the rotarod in non-ischemic mice (Table 1). The time of walking on the rotarod in ischemia plus vehicle group \((n=10)\) was significantly lower than sham groups \((p<0.01)\) at all time points following stroke, and reduced by 43.28%, 36.64%, and 31.26% at day 1, 3, and 7 after surgery, respectively, compared with pre-surgery, indicating animal model of ischemic stroke was successful (Fig. 2A). Progesterone significantly increased the ability of remaining on the rotarod at all time points within 7 days after ischemia in comparison with ischemia plus vehicle animals \((n=10, p<0.01)\), and the percentage difference between pre- and post-surgery were 26.85%, 7.70%, and 3.57% at day 1, 3, and 7 after stroke (Fig. 2A). There was significant improvement in behavioral performances in rehabilitation training group at all time points after ischemia \((n=10, p<0.01)\) (Fig. 2A). Compared with pre-surgery performance, the latency to fall decreased by 35.64%, 11.92%, and 8.32% at day 1, 3, and 7 after surgery, respectively. There was no significant difference from day 2 to day 7 after ischemia between progesterone group and...
rehabilitation training group (Fig. 2A). Combination with progesterone and rehabilitation training has further improved the ability of remaining on the rotarod at all time points within 7 days after ischemia ($n = 10$, $p < 0.01$), and the percentage difference between pre- and post-surgery were 20.99%, 10.68%, and 0.59% at day 1, 2, and 3 after stroke, respectively (Fig. 2B). Combination therapy significantly enhanced rotarod performance at all time points compared with rehabilitation training alone ($p < 0.05$), and enhanced rotarod performance was also observed in combination therapy group from day 2 after stroke compared with progesterone alone ($p < 0.05$, Fig. 2B). These data suggested that combination therapy has exerted a neuroprotective effect in improving coordination after ischemia.

We then evaluated the effect of the progesterone and rehabilitation training on grip strength. The grip strength of mice in sham had a slight decrease at the first day after surgery due to surgery effect, and then returned to pre-surgery level on the second day after surgery. There was no significant difference between sham controls (vehicle, $n = 10$; progesterone, $n = 8$; rehabilitation, $n = 10$) at different time points within 7 days post-surgery, suggesting that progesterone or rehabilitation has not significantly increased the forelimb grip strength in non-ischemic mice (Table 2). As expected, ischemia induced a significant reduction in grip strength of the forelimbs, and the grip strength decreased at all time points in animals subjected to ischemia plus vehicle ($n = 10$) (Fig. 2C). The forelimb grip strength was reduced by 30.38%, 35.97%, and 28.32% at day 1, 3, and 7 after surgery, respectively, compared with pre-surgery. Progesterone treatment significantly improved forelimb grip strength at different time points within 7 days after ischemia compared with ischemia plus vehicle group ($n = 10$, $p < 0.01$) (Fig. 2C). The percentage difference between pre- and post-surgery was 23.67%, 19.62%, and 13.40% at day 1, 3, and 7 after surgery, respectively. The forelimb grip strength in rehabilitation training group was increased significantly from day 3 to day 7 after ischemia compared with ischemia plus vehicle group ($n = 10$, $p < 0.05$) (Fig. 2C). The reduction in forelimb grip strength was 28.42%, 27.15%, and 19.55% at day 1, 3, and 7 after surgery, respectively, compared with pre-surgery. Progesterone significantly improved forelimb grip strength at all time points compared with rehabilitation training alone at all time points within 7 days ($p < 0.01$) (Fig. 2D). Improved recovery of grip strength was also observed in combination therapy group from day 2 compared with progesterone alone ($p = 0.05$) (Fig. 2D). In addition, progesterone and rehabilitation training had synergistic effect (combined effect is greater than the sum of individual effects of progesterone and rehabilitation training) on improving forelimbs grip strength at day 2 after ischemia (grip strength was improved by 18.80%, 5.24%, and 28.44% for progesterone, rehabilitation training, and progesterone + rehabilitation training, respectively, compared with pre-surgery).
terone may exert neuroprotective effects by acting on largely unclear. Previous studies suggest that progesterone in ischemic stroke remains and neurological deficit. The detailed neuroprotective injection was effective in reducing infarct volume and neurological deficit. Most studies have administered progesterone at 1 h after ischemia. Considering the time interval addition, most studies have administered progesterone at a dosage of 2 mg/kg, lower than previous reports. Our results indicated that progesterone administered at a dosage of 2 mg/kg after stroke via subcutaneous injection was effective in reducing infarct volume and neurological deficit. The dosage of 2 mg/kg was selected to facilitate observing accumulating effect of combina-
tion therapy. In addition, low dosage of progesterone can alleviate withdraw effect induced by high dosage (Cutler et al., 2006b). Progesterone administered subcutaneously may be slowly released and maintained at steady plasma concentration (Cutler et al., 2006a). In addition, most studies have administered progesterone at 1 h after ischemia. Considering the time interval between stroke onset and the initiation of treatment is normally longer than 1 h, we administered progesterone at 4 h after ischemia in our experiments. Our results showed that progesterone administered at a dosage of 2 mg/kg at 4 h after stroke via subcutaneous injection was effective in reducing infarct volume and neurological deficit. The detailed neuroprotective mechanisms of progesterone in ischemic stroke remain largely unclear. Previous studies suggest that progesterone may exert neuroprotective effects by acting on multiple-targets. Progesterone can reduce glutamate excitotoxicity, attenuate inflammation cascade damage (Jung et al., 2009; Wang et al., 2011), decrease apoptosis by PI3K/Akt pathway (Ishrat et al., 2012), and reduce both vasogenic and cytotoxic edema as well as prevent additional secondary neuronal loss (Meffre et al., 2005). In addition, progesterone may also repair the blood-brain barrier (Ishrat et al., 2010) and suppress microglial and astroglial activation (Labombarda et al., 2011).

We used hanging wire training, rotarod training and climb ladder training as the rehabilitation tasks. Hanging wire training can compel mice to use injured limb to grip wire and move. The therapy can promote functional recovery of the impaired limb (Nudo & Friel, 1999). Climbing ladder is a complex task for the whole motor system. Climbing requires precise reaching movements of hands and feet to be able to hold the grids. The training may improve balance, body stabilization, and the simultaneous coordination of all four limbs (Marianne Anke et al., 2011). Accelerated rotarod training requires running speed coinciding with rod rotating speed to avoid falling, and is considered to be a valid paradigm for motor skill learning (Buitrago et al., 2004). The training involves whole body exercising and can promote balance and coordination ability. After rehabilitation training, brain function recovery was improved in mice following stroke, indicating the methods of rehabilitation training were effective.

In the present study, we evaluated the effects of progesterone, rehabilitation training and their combination on infarct volume and behavioral outcomes after acute ischemic stroke. Consistent with previous studies (Chen et al., 1999; Gibson et al., 2011; Gibson & Murphy, 2004; Ishrat et al., 2009; Jiang et al., 1996; Kunon et al., 2000; Sayeed et al., 2007), we found that progesterone reduced infarct volume and neurological deficits. Most studies showed that progesterone administered intraperitoneally at a dose of 8 mg/kg at 1 h, 6 h, 24 h and 48 h after ischemia is effective in reducing infarction and neurological deficit (Gibson et al., 2011; Gibson & Murphy, 2004; Sayeed & Stein, 2009; Sayeed et al., 2007), and two studies indicated that progesterone at a dosage of 4 mg/kg has maximal beneficial effect on the survival of newborn neurons in the hippocampal dentate gyrus of adult mice (Zhang et al., 2010a; Zhang et al., 2010b). We administered progesterone at a dosage of 2 mg/kg, lower than previous reports. Our results indicated that progesterone administered at a dosage of 2 mg/kg after stroke via subcutaneous injection was effective in reducing infarct volume and neurological deficit. The dosage of 2 mg/kg was selected to facilitate observing accumulating effect of combination therapy. In addition, low dosage of progesterone can alleviate withdraw effect induced by high dosage (Cutler et al., 2006b). Progesterone administered subcutaneously may be slowly released and maintained at steady plasma concentration (Cutler et al., 2006a). In addition, most studies have administered progesterone at 1 h after ischemia. Considering the time interval between stroke onset and the initiation of treatment is normally longer than 1 h, we administered progesterone at 4 h after ischemia in our experiments. Our results showed that progesterone administered at a dosage of 2 mg/kg at 4 h after stroke via subcutaneous injection was effective in reducing infarct volume and neurological deficit. The detailed neuroprotective mechanisms of progesterone in ischemic stroke remain largely unclear. Previous studies suggest that progesterone may exert neuroprotective effects by acting on increasing forelimb grip strength after ischemia.

4. Discussion

In line with previous studies, our data suggested that rehabilitation training improved behavioral outcome and reduced infarct volume following stroke (Matsuda et al., 2011; Zhang et al., 2012c). However, the degree of reduction in infarct volume and the improvement of functional recovery in the rehabilitation training group as assessed by grip strength test were smallest among three groups. But in rotarod test, progesterone has not further improved coordination ability compared with rehabilitation. The neuroprotective mechanism of rehabilitation training after brain injury is not fully elucidated but may be related to reorganization in the adjacent intact cortex (Nudo et al., 1996; Traversa et al., 1997) and circuit plasticity in ipsilateral and contralateral cortex (Gonzalez Andino et al., 2011; Shepherd, 2001). The adult central nervous system has a substantial plastic capacity after injury (Cohen et al., 1998; Zhang et al., 2005). Lost functions can be compensated for or relearned after stroke by task-specific practice (Nudo & Friel, 1999).
Evidences suggested that ischemic injury can induce an increase in spine turnover rate and synaptogenesis (Bury & Jones, 2002; Jones et al., 1999) and enhance neurogenesis (Kee et al., 2001; Nudo et al., 1996) in the non-damaged cortical regions. The rebuilding of the synaptic circuits in the injured brain may be enhanced by post-injury rehabilitation training such as motor training and experience in a complex environment (Briones et al., 2005; Nudo et al., 1997). Rehabilitation training such as constraint-induced movement therapy appears to be mediated mainly through compensatory strategies rather than a decrease in impairment (Zhuo et al., 2013). Such therapy can promote behavioral recovery by increasing motor cortex reorganization and neurogenesis after stroke (Fraser et al., 2002; Gauthier et al., 2008; Liepert et al., 1998; Sawaki et al., 2008). Motor-skill training after cortical injury is able to promote reorganization of cortical maps in tissues adjacent to the injury and is associated with motor recovery (Kopp et al., 1999). In addition, the neuroprotective effect of early exercise may also be related to regulating mitochondrial biogenesis by modulating the expression of mitochondrial-specific transcription factors, such as PGC-1, NRF-1 and COX IV (Bayod et al., 2011; Steiner et al., 2011).

In the present study, we observed that combination therapy further enhanced functional outcome after ischemic stroke, but combination therapy had no additive effect in reducing infarct volume. Though some studies argued that lesion size is closely related to neurological deficits (Rogers et al., 1997), this is certainly not always the case (Wahl et al., 1992). Consistent with our results, studies have suggested that progesterone can improve functional recovery without reducing infarct size (Gibson et al., 2011; Hattori et al., 2000). Lemmens and his colleague found that reducing the levels of EphA4 does not reduce infarct size but can improve functional recovery (Lemmens et al., 2013). Thus, functional recovery may be associated with multiple aspects of the injured cortex.

In our study, we used phototherapeutic model to evaluate the effects of progesterone and rehabilitation training. Although phototherapeutic model has many advantages (eg. targeted lesion), it also has limitations. Rose bengal induced phototherapeutic is generally difficult to reperfuse, and may have different pathological progression than other models. Thus, the neuroprotective effect we observed here need to be verified in other models. In addition, we only assessed the effect of combination therapy within one week. Further studies are warranted to assess the long-term efficacy of progesterone and rehabilitation training following stroke.

In conclusion, our results indicated that progesterone, rehabilitation training and their combination had neuroprotective effect in reducing infarct size, but we found that there was no additive effect between progesterone and rehabilitation training in the reduction of infarct volume. However, we found that combination of progesterone and rehabilitation training has enhanced efficacy in improving functional recovery, suggesting that different mechanisms, other than reduction of infarct volume, were involved in promoting functional recovery. The mechanisms underlying the enhanced efficacy need to be explored further in future studies.

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499


