International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 7 (1): 50-57, 2011
ISSN 1811-7775 / DOI: 10.3923/ijp.2011.50.57
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Effects of Wuling Capsule on Hippocampaldependent Cognitive Changes in Post-stroke Depression Rats

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Abstract: Post-Stroke Depression (PSD) always leads to various cognitive deficits and many unfavorable outcomes. The aim of this study was to examine the protective effects of Wuling capsule on changes of hippocampal-dependent learning and memory and expression of Brain-Derived Neurotrophic Factor (BDNF) in hippocampus. Adult male Sprague-Dawley rats were exposed to a 3-week chronic mild stress paradigm after ischemic surgery. The level of depression was assessed with sucrose preference and the morris water maze was introduced to detect the changes of learning and memory. The BDNF expression was examined by immunohistochemical staining and the assay of mRNA amplification using semi-quantitative reverse transcription-PCR. A significant decrease in the sucrose preference and BDNF expression was found in the model rats. Escitalopram was administrated orally at a dose of 0.2 mg kg $^{-1}$ b.wt. day for 21 days. The depressed behavior was improved and the cognitive deficits recovered to normal level. Moreover, the low expression of BNDF increased significantly. The same changes were found in Wuling capsule treatment at the dose of 100 mg kg $^{-1}$ b.wt. except that BDNF expressions remain low after treatment. These results indicate that the mechanisms of Wuling capsule improving the impairments of learning and memory in PSD rats are not mediated by BDNF.

Key words: Post-stroke, depression, BDNF, learning and memory in rats

INTRODUCTION

Post-Stroke Depression (PSD) is a common comorbidity after stroke with the prevalence ranging from 25 to 79% (Thomas and Lincoln, 2008). Recently epidemiological researches, clinical observations, neural imaging and neuropathologic have studied the relationship of depression and stroke and manifested the existence of complex interactions. For example, PSD may be partly one result of stroke, but in turn it can be one reason for further accidents of brain vessels (Liebetrau *et al*., 2008). Many psycho-social factors involving in the occurrence of PSD are becoming more interesting and arousing greater attention to many clinical researchers. But one challenge that still has to be faced to seriously is how to solve these various confounding factors perfectly. Animal research can overcome this drawback and be conducive to well understanding the etiopathogenisis of PSD by precisely controlling the confounding factors.

Thirty five to eighty two percent of stroke patients were found to have cognitive impairments in at least one domain which might influence the clinical outcome negatively and increase economic burden (Nokleby *et al*., 2008; Donovan *et al*., 2008). Cognitive function is an important predictor of successful rehabilitation after stroke, for even small cognitive deficits can lead to considerable functional problems and may hinder both the rehabilitation process and secondary stroke prevention (Duits *et al*., 2008). The underlying mechanisms of cognitive impairment in PSD remain still poorly understood. Cogitive deficits might reflect decreased plasticity of hippocampus. BDNF not only regulates neurogenesis in the hippocampus, but could act as a basis of disposition in patients suffered from depressive disorder (Taliaz *et al*., 2010). It has been reported that BDNF and neural plasticity of hippocampus may be the targets for antidepressants (Castren and Rantamaki, 2010). Escitalopram as a first-line antidepressant has shown the effect of improving cognitive function partly by increasing the BDNF expression (Bjornebekk *et al*., 2008). Wuling capsule, a traditional Chinese medicine, which has been used in clinic for many years, has been proved to be fully potent in improving the signs of insomnia and cognitive deficits since its introduction in 1999. Recently, it is shown that a more powerful response rate

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of anti-depression has been better when Wuling capsule was administered in combination with other first-line antidepressants than when either it or any other antidepressant was given alone, which indicates the pharmacological action of Wuling capsule is worthy of being further studied. We aimed to test the assumption that the process of Wuling capsule improving the cognitive deficits in PSD rats is associated with the change of BDNF expression in hippocampus. In this report the antidepressant Escitalopram will be used as a positive reference to observe the change of BNDF expression and cognition in PSD rats.

MATERIALS AND METHODS

Animals and experimental procedures: Forty adult male Sprague-Dawley strain rats (250-300 g) were chosen from the animal center of The First Affiliated Hospital of Zhejiang University in 2009 and were divided randomly into four groups (n = 10): Control group; Model group; Escitalopram group and Wuling group. Before experiment all rats were housed in colony cages at an ambient temperature of 25±2°C and 50-70 % relative humidity, with 12 h light: dark cycle. The animals were given free access to standard pellet chow and drank water. When the basal sucrose preference test was over, rats from the model and the two drug treating groups underwent cerebral ischemic operation and then were submitted to the 21 day procedures of Chronic unpredictable Mild Stress (CMS) and living alone. Along with the course of CMS, Escitalopram tablets (Xi'an-Jianssen Pharmaceutical Ltd,. China) and Wuling capsules (Zhejiang Zhuoli Medicine Company, China) were added to their diets of the corresponding rats at the dose of 0.2 mg kg^{-1} b.wt. and 100 mg kg^{-1}, respectively. On the day of 7, 14, 21 of CMS procedures, sucrose preference were calculated. Followed by the last sucrose Preference test, all the animals were subject to the Morris Water Maze Test, then were put to death and their brain samples were isolated for BDNF immunohistochemistry assay. All experimental procedures were approved by the Ethics Committee for Experimental Animal Research of the University of Zhejiang and all efforts were made to minimize animal suffering and to reduce the number of animal use.

Modeling

Surgical procedure: Selective Middle Cerebral Artery (MCA) occlusion with intraluminal suture was used for modeling in model and drug treating rats. Ten percent chloral hydrate was injected peritoneally at a dose of 0.3 mL 100 g^{-1} for anesthesia. A cervical midline incision was made and the left carotid artery and branches were isolated. After exposure and ligation of the Common Carotid Artery (CCA), the left external carotid artery and pterygopalatine artery were opened. An aneurysm clip was placed on the proximal internal carotid artery, while an arteriotomy was made on the distal common carotid artery. An uncoated 25 mm long segment of 3-0 nylon monofilament suture with the tip rounded by wax was inserted into the arteriotomy. The aneurysm clip was removed and the suture was advanced under direct visualization into the internal carotid artery approximately 19 to 20 mm from the bifurcation to occlude the ostium of the MCA. The rats developed typical symptoms and pathological manifestations after the surgery. Twelve to twenty four hour later after the surgery, neurologic outcomes were scored on a 0-5 grading scale. 0 means no nerve injury; 1 means inability to fully stretch frontal claws; 2 means circling to the opposite side; 3 means tumbling to the opposite side; 4 means unable to walk spontaneously and unconsciousness. The occluding suture was kept in place for 20 min. At the end of the ischemic period, the suture was removed and the surgical incisions were closed. The animal was allowed to recover and then transported to the intensive care unit at the animal facility for postoperative monitoring. Rats scored 0 or 4 or dead for any other reasons were abolished.

CMS paradigm: One week later after the surgery, the animals were subjected to the CMS procedures to induce the core depressed symptom: anhedonia. The CMS protocol was designed to maximize the unpredictable nature of the stressors according to previous studies (Nirmal *et al*., 2008) with a minor modification. One of the following stressors was administered daily (in random order) over a period of 3 weeks: fasting for 20 h; water deprivation for 17 h; swimming at 4°C for 5 min; high temperature environment (40°C) for 5 min; 45° cage tilt for 17 h; shaker stress (horizontal shakes at high speed) for 10 min; being restraint for 2 h; soiled cage (200 mL water in 100 g sawdust bedding) for 5 h; persistent illumination (light for 17 h); tail pinch for 2 min and unpleasant noise for 5 min. Immediately after each stress session, the rats were returned to the solo room and situated in standard conditions until the next session of the CMS regime.

Sucrose preference testing: Sucrose preference has been proposed to detect the levels of anhedonia and the procedures have been described by Orsetti *et al*. (2007). Animals were housed individually with two bottles containing either 1% sucrose solution or tap water with standard lab chow available continuously. The basal sucrose preference was obtained prior to the punishment of CMS and then sucrose preference was calculated three

times under the similar conditions (two-bottle test, 2 h periods) on every weekend since the beginning of the CMS procedures.

Hippocampal immunohistochemistry assay: Immunohistochemistry was performed under the same conditions in each group in order to ensure the accuracy of immunohistochemical staining. SABC-kit (BOSTER Corp) was applied for BDNF immunohistochemistry assay. After dewaxing and hydration, sagittal paraffin sections with the thick 3 um were closed by 5% BSA closing for 20 min. Followed by sequential incubations with Rabbit-anti-mouse BDNF (1:200, Sigma) (4°C) overnight and biotinylated goat-anti-rabbit IgG and streptavidin-biotin peroxidase complex (SABC) for 2 h (37°C), the brain slices were colored with 3,3'-diaminobenzidine. The sections were mounted in neutral resin following hematoxylin-restaining and dehydration and visualized under an Olympus light microscope (OLYMPUS Shanghai Trading, China). The numbers of $BDNF^+$ cells were counted and analyzed in the same part of the CA3 zone.

Morris water maze training and testing: Morris Water Maze (MWM) was employed to assess the learning and memory of rats. The MWM procedure was based on a principle (Harvey *et al*., 2008): as the animals were placed in a large pool of water and they dislike swimming, their tendency was to escape from the water being accomplished by finding an escape platform. MWM consisted of a large circular pool (150 cm in diameter, 45 cm in height), filled to a depth of 30 cm with water at 27±1°C. The water was made opaque with non-toxic black colored dye. The tank was divided factitiously into four equal quadrants. A submerged platform (9 cm in diameter) painted in white was placed inside the target quadrants of this pool, 1 cm below surface of water. The position of platform was kept unaltered throughout the training session. Each animal was subjected to four consecutive trials on each day with a gap of 15 min. The rat was gently placed in the water of the pool between quadrants, facing the wall of pool with drop location changing for each trial and allowed 120 sec to locate submerged platform. Then, it was allowed to stay on the platform for another 15 sec. If it failed to find the platform within 120 sec, it was guided gently onto platform and allowed to remain there for 15 sec. Escape Latency Time (ELT) to locate the hidden platform in water maze was noted as an index of acquisition or learning. Each animal was subjected to four acquisition trials daily for four consecutive days. On the fifth day, the platform was removed and each rat was allowed to explore the pool for 120 sec. The average time taken in all four quadrants was noted. The mean time taken by the animal in target quadrant searching for the hidden platform was noted as an index of delayed recall. A video camera above the centre of the pool was connected to a computerized tracking system that recorded and analyzed animal behavior (Beijing Sunny Instruments Co. Ltd, China).

RT-PCR: The samples of 5 ug total RNA were extracted by Trizol reagent (Gibco Brl, Rockville, Massachusetts, USA) and reversely transcribed into cDNA based on a typical protcol. Briefly, BDNF mRNA expression was determined through a semi-quantitative PCR with β-axtin as a reference. RT-PCR primers were designed as follows:

- **β-actin forward:** 5'-CAACTTGATGTATGAAGGC TTTGGTGCTGA-3'
- **Reverse:** 5'-ACTTTTATTGGTCTCAA GTCAGTGT ACAGC-3'
- **BDNFforward:** 5'-AAAACCATAAGGACGCGGAC TTCCT-3'
- **Reverse:** 5'-AAAGAGCAGAGGAGGCTCCAGAT-*3*

The size of products including β-actin (328 bp) and BDNF (536 bp) was adequate to match and incorporate target genes. The PCR reaction mixture (specific primers, reaction buffer, reverse transcriptase and dNTP) was submitted to 30 amplification cycles. The PCR cycling contained the following steps: preincubation of 2 min at 37°C, denaturation of 5 min at 94°C, annealing of 45 sec at 57°C, elongation for 10 min at 72 and 45°C for 15sec. Then, 2% agarose gel electrophoresis was applied to confirm the mRNA expression of RT-PCR products (10 uL). The signal intensities were quantified by Dolphin software (Wealtec Corp).

Statistical processes: All data were described as Mean±SD and were dealt with software SPSS15.0. One way ANOVA was performed and the mean comparisons in mutigroups were accomplished using the SNK-q test. The standard for statistical significance was $p<0.05$.

RESULTS

Comparison of sucrose preference: To investigate the effects of Wuling capsule on depression behavior, we examined the depression-related rewarding behavior in sucrose preference test (Fig. 1). Quantitative analysis indicated that sucrose preference was significantly decreased from 1 to 3 weeks after CMS in model rats, in contrast a significant increase was found after drug treatment using either escitalopram or Wuling capsule

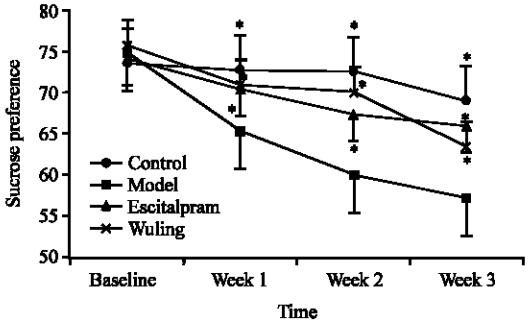

Fig. 1: Comparison of sucrose preference among four groups. (1) Control (n = 10), (2) Model (n = 7), (3) Escitalopram (n = 8) and (4) Wuling (n = 7). One-way ANOVA and SNK-q test were used, *p<0.05, vs. Model group

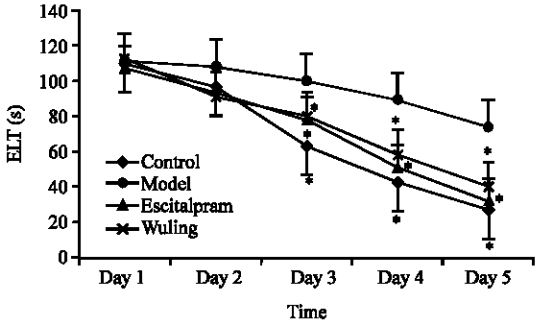

Fig. 2: Comparison of escape latence time (ELT) among four groups. (1) Control (n = 10), (2) Model (n = 7), (3) Escitalopram (n = 8) and (4) Wuling (n = 7). One-way ANOVA and SNK-q test were used, *p<0.05, vs. Model group

and there was no difference in escitalopram group and Wuling group, suggesting that Wuling capsule acting as an antidepressant has similar efficacy to escitalopram.

The evaluations of learning and memory: MWM test was used to evaluate the learning functions in this research (Fig. 2). And memory functions were also evaluated in this research (Fig. 3a, b). The ELT, as a learning measurement was found decreased significantly after the surgery and CMS procedures. This low ELT could be reversed by escitalopram or Wuling capsule treating. Comparison of memory functions using two appraisal measurements, duration in target quadrant and spanning platform frequency showed the similar results that the low remembrance in model rats was recovered to the normal level after escitalopram or Wuling capsule intervention and no significant difference was shown in their efficacys.

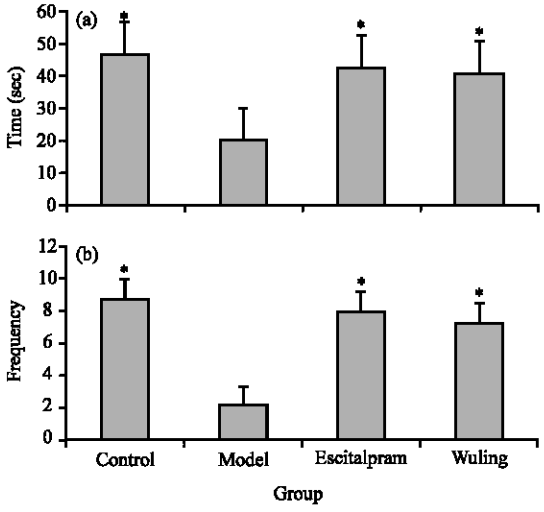

Fig. 3: The analysis of delayed recall from four groups. The left statistical map shows the comparison of time in target quadrant and the right bar graph reveals the frequency of spanning the position of the platform stay. (1) Control (n = 10), (2) Model (n = 7), (3) Escitalopram (n = 8) and (4) Wuling (n = 7). One-way ANOVA and SNK-q test were used, *p<0.05, vs. Model group

These results indicate that Wuling capsule as well as escitalopram may play an important role in enhancing cognitive impairments in PSD rats.

The expression of hippocampous BDNF and BDNF$^+$ cell count: To investigate the BDNF changes in CA3 zone, hippocampal sections were detected by SABC-immunohistochemical assay. A large number of the brown patches in the sections demonstrated BDNF positive expression. The brown kytoplasm surrounding the black nuclei represented the BDNF$^+$ cells (Fig. 4a-c). We counted the number of BDNF-positive cells and found that the products of BDNF expression were affluent in control group, but meager in PSD group. After escitalopram treating, the number of BDNF$^+$ cells increased markedly and became normal, whereas no improvement was found in Wuling treatment in PSD rats. These results revealed that not Wuling capsule but escitalopram could restore the low BDNF expression induced by PSD in CA3 zone.

The changes of BDNF mRNA in hippocamous: We found low mRNA expression of BDNF in hippocampous in PSD rats. Escitalopram significantly reversed the decline of BDNF mRNA expression, but no significant change was found in Wuling capsule treatment (Fig. 5).

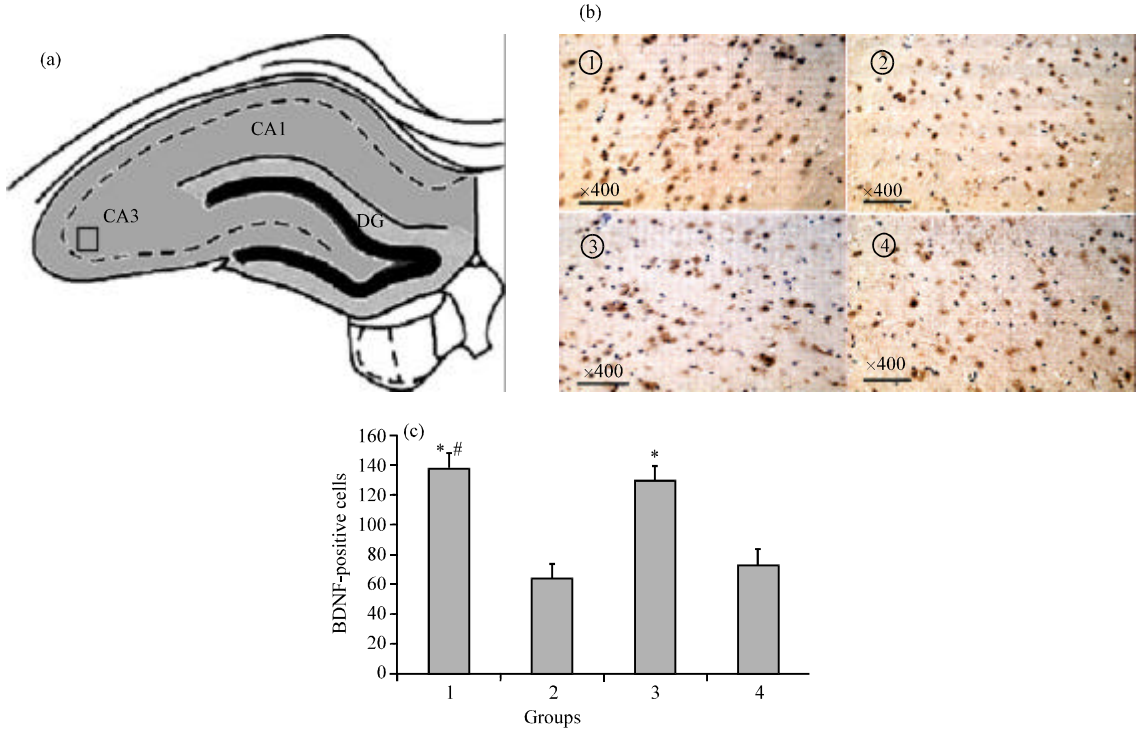

Fig. 4: Expression of BDNF and count of BDNF-positive cells in hippocampal CA3 zone among four groups. (a) Hippocampal structural diagrammatic view showing the locations of CA3, CA1 and Dentate Gyrus (DG) and the counting position (panel), (b) Photograph of BDNF expression detected by SABC-immunohistochemical staining (Light microscopy, ×400, bar = 250 μm) and (c) Comparison of BDNF-positive cells. Data were represented as $\bar{x}\pm s$, *p<0.05, vs. control group; #p<0.05, vs. Wuling group. (1) Control (n = 10), (2) Model (n = 7), (3) Escitalopram (n = 8) and (4) Wuling (n = 7)

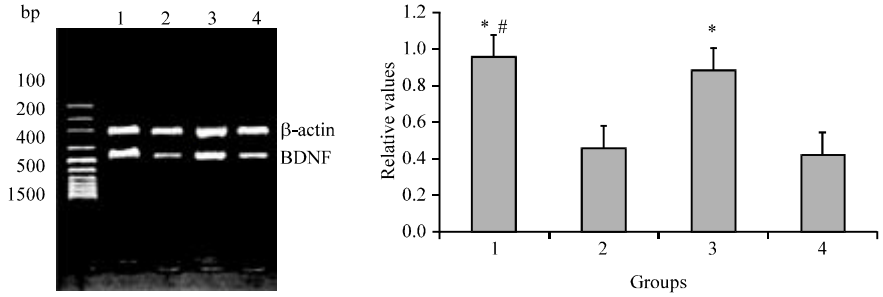

Fig. 5: Expression of BDNF mRNA in hippocampus in four groups. The left graph is 2% agarose gel images showing specific BDNF and β-actin mRNA expressions and the right bar graph was results of relative intensity of BDNF mRNA to β-actin mRNA. Data were represented as $\bar{x}\pm s$, *p<0.05, vs. control group; #p<0.05, vs. Wuling group

DISCUSSION

Chronic unpredictable mild stress and living alone following selective middle cerebral artery embolization was a most commonly used animal model underlying PSD, for its high validity to imitate the depressed symptoms of stroke patients and to be easily detected by sucrose preference test and open field trial. Sucrose preference test on PSD rats showed the level of response to rewards, reflecting the core depressed symptom, anhedonia. In this study, we administered Wuling capsules to PSD model rats and examined the sucrose

reference, hippocampous-dependent cognitive functions and expression levels of BDNF. Present results showed reduced sucrose preference recovered to the normal level indicating its anti-depression effects.

Morris water maze test is a classic approach to evaluate the hippocampus-dependent learning and memory functions in animal models. Because hippocampus accepts a large number of afferent connects from different cortical and subcortical structures and releases nerve impulses through pyramidal neurons in CA3 zone, it participates in spatial learning and informational retention and the process to transform the new information into short-term memory. Multiple risk factors such as stroke, chronic stress and lack of social support may influence the hippocampal formation to induce cognitive impairments. In this report the learning and memory deficits due to PSD were confirmed. Furthermore, Wuling capsule as well as escitalopram may play an important role in enhancing cognitive impairments in PSD rats. Although, the mechanism of animals' learning and memory is not fully elucidated, there is still a certain relationship with the expression of BDNF in hippocampus (Harvey *et al*., 2008). BDNF, a neurotrophin, is known to promote neuronal differentiation stimulating neurite outgrowth in the developing CNS and is also known to modulate synaptic plasticity, thereby contributing to learning and memory in the mature brain (Nakajo *et al*., 2008). Several studies have been confirmed the relationship of the BDNF and the performances of Morris water maze test as follows: Pregestational stress reduce the expression of BDNF in CA3 area in the hippocampus of offspring, further resulting in the impairment of memory in the adult offspring (Huang *et al*., 2010). The Ventral Subicular Lesioned (VSL) rats with H3-GFP transplants showed enhanced expression of BDNF in the hippocampus and performed well in Morris water maze tasks (Rekha *et al*., 2009). Exercise upregulates BDNF within the hippocampus and is associated with an enhancement of cognitive recovery after a lateral fluid percussion injury (Griesbach *et al*., 2009). It has been reported that BDNF may exert its protective effect on nervous regeneration, reverse synaptic loss and stabilize gene expression and improve the cell signal transduction to restore learning and memory impairments (Nagahara *et al*., 2009). Antidepressant action may involve stimulation of BDNF and target the biochemical pathways of hippocampal plasticity and normalize the abnormal hippocampal function to improve cognition, for instance, escitalopram treatment was able to increase BDNF mRNA levels in leukocytes (Cattaneo *et al*., 2010) and its efficacy was related to the Val66Met polymorphism in the BDNF gene (Rajewska-Rager *et al*., 2008). Escitalopram, a selective serotonin reuptake inhibitor, may influence several physiological processes in the hippocampus, such as, elevating intracellular cAMP levels and enhancing neurogenesis partly through increasing the expression of BDNF. In our research, it was confirmed repeatedly that escitalopram increased the expression of BDNF and improved the deficits of learning and memory. Additionally, an interesting phenomenon was found that Wuling capsule exerted similar effects like escitalopram to improve depression and cognitive impairments, but could not restore hippocampal abnormal BDNF expression in PSD rats. On one hand, in addition to the target of BDNF, other substrates such as the hypothalamus - pituitary - adrenal axis and various kinds of immune cytokines may involve in the injury and repair; on the other hand, Wuling Capsule contains adenosine, polysaccharides, steroid and 19 kinds of amino acids such as glutamate and gamma-aminobutyric acid (GABA). Animal studies have demonstrated that Wuling capsule could significantly enhance the sedative effect on neural tissues and promote glutamate to be uptaken quickly in the central nervous system. These remarkable effects are mainly produced by enhancing the activity of glutamic acid decarboxylase to increase GABA synthesis and improving the potency of GABA receptor binding in cerebral cortex, whereas escitalopram reacts mainly through the serotonin receptor. GABA, the main inhibitory neurotransmitter in the adult brain, may interact with BDNF expression mediating the neural network development, for example, activation of metabotropic GABA(B) receptors triggers the secretion of BDNF and promotes the development of perisomatic GABAergic synapses in the newborn mouse hippocampus (Fiorentino *et al*., 2009). BDNF inversely enhanced the expression of GABA receptors in cultured spiral ganglion neurons may have important implications for neural development and plasticity (Sun and Salvi, 2009). It has reported that dendritic BDNF release in CA3 pyramidal neurons causes long-term potentiation of the frequency of GABAA receptor-mediated spontaneous postsynaptic currents and this mechanism is thought to play a role in the pathophysiology of learning and memory deficits in fetal alcohol spectrum disorder (Casarotto *et al*., 2010). Here, we presume GABA- and 5-HT-related mechanisms may equally contribute to the similar behavioural/cognitive effects of the anti-depressants. This is why Wuling capsule can improve various memory deficits in this situation as well as in some other unfavorable situations such as hypoxia and fatigue.

CONCLUSIONS

Wuling capsule has been shown to improve the cognitive deficits and response rate of anti-depression. A series of tests were performed to investigate the effects of Wuling capsule on the cognitive impairment in PSD rats by comparing with escitalopram. The results of sucrose preference and learning and memory tests confirm the role of Wuling capsule in improving the cognitive impairment in PSD rats, but may be through different mechanisms.

ACKNOWLEDGMENTS

This work was supported by two Foundations from Medical Project of the Health Department of Zhejiang Province (No. 2008A072) and Project of Traditional Chinese Drug Administration Bureau of Zhejiang Province (No. 2009CB035).

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