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# Neuroprotective Effect of Escitalopram Oxalate in Rats with Chronic Hypoperfusion

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**Summary:** The neuroprotective effects of escitalopram oxalate in rats with chronic hypoperfusion and the possible mechanism were explored. Chronic hypoperfusion (2-VO) model was prepared and given escitalopram oxalate (experimental group) or PBS (control group) after 6 weeks. Eight weeks after the operation, Morris water maze test was carried out to evaluate the learning and memory ability of the rats. The cell proliferation, three-dimensional vascular distribution, cell morphological changes in ischemic area and the plasma vascular endothelial growth factor (VEGF) were detected to explore the possible mechanisms. (1) Morris water maze test showed that the escape latency in the experimental group was significantly shorter than in the control group, while the first quadrant swimming time in the experimental group was significantly longer than the control group (both $P<0.01$). (2) Cerebrovascular confocal detection results showed that the inside diameter of capillaries was significantly less in the experimental group than in the control group; the vascular density was significantly increased in the experimental group and the total area of capillaries was also significantly increased in the experimental group as compared with the control group. (3) There was statistically significant difference in BrdU-positive cells in the ischemic brain tissue between the experimental group and the control group ($P=0.003<0.01$). (4) VEGF concentrations in the plasma and the ischemic area were higher in the experimental group than in the control group ($P<0.05$). It was concluded that escitalopram oxalate could significantly improve the learning and memory ability of the rats with chronic cerebral ischemia probably by the VEGF-mediated angiogenesis.
**Key words:** escitalopram oxalate; chronic hypoperfusion; neuroprotection; mechanism; vascular endothelial growth factor

Cerebrovascular disease is the leading life-threatening cause that severely damages health of human being among three major diseases. It is characterized by high incidence, high mortality and high disability rate. Ischemic cerebrovascular disease (ICD) accounts for about 80% of cerebrovascular disease[1]. Although a lot of progress has been made on study of ICD, effective intervention is still not available, probably due to complex mechanism involved in cerebral ischemic injury[2]. Recent studies demonstrated selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine and paroxetine not only improved depression in patients who had brain attacks but also facilitated prognosis of neurological function and protected nervous tissue[3, 4]. The protection of SSRIs on nerves occurs via mechanisms that involve: (1) improvement of depression and enhancement of neurological function recovery after stroke[5, 6]; (2) promotion of angiogenesis and neurogenesis in ischemic area; (3) alleviation of neuronal injury and apoptosis; (4) promotion of synapses recovery; and (5) other aspects.

Escitalopram oxalate (CIT), S-(+)-1-[3-(dimethylamino) propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofuran-carbonitrile oxalate, is a selective serotonin reuptake inhibitor. It is an active S (+)-enantiomer present in the racemate citalopram. As a new SSRI, CIT shows greater efficacy[7], better tolerance and less adverse reactions[8] than citalopram. Recent studies about SSRIs have focused on their neuroprotective effect on ICD, especially on acute ICD. However, the use of SSRIs in chronic hypoperfusion has been seldom reported, much less for the protective effect of CIT on chronic hypoperfusion.

2-VO is a typical rat model of chronic cerebral hypoperfusion well suited for fundamental research, which mimics a series of pathological and physiological process of vascular dementia caused by ischemia and anoxia in brain tissues. In the present study, the rat 2-VO model was established and then intervened with CIT. Two consecutive weeks after intervention, the changes of spatial learning and memory were observed in rats. The effects of CIT on blood vessels of ischemic brain tissues were analyzed by observation of three-dimensional imaging of brain blood vessel as well as proliferation and differentiation of cells in ischemic area in the brain. The expression of vascular endothelial growth factor (VEGF) was also determined for the analysis of potential molecular biological mechanism, aiming at providing evidence for use of CIT in the treatment of chronic ICD.

## 1 MATERIALS AND METHODS

### 1.1 Establishment of Animal Model and Assignment

Sixty male SPF Sprague-Dawley (SD) rats, 8 to 12 weeks of age, weighing 220–250 g, were purchased from the Animal Experimental Center of Wuhan University

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(China) [certificate number: SCXK(Hubei)2009-0005]. These mice were fed in the Animal Experimental Center of Wuhan University and housed at a humidity of 40%–60% with standard rodent diet. The amount of food and water consumption was dynamically observed. The bedding in the cage was replaced once each day. All experiments were performed in compliance with the Guideline on Animal Care of the Ministry of Science and Technology.

The 2-VO model was prepared 5 days after acclimation to restraining cages. The rats were fasted for food but had free access to water 12 h before operation. 10% chloral hydrate (0.35/kg) was injected intraperitoneally for the induction of anesthesia. After standard skin disinfection and preparation, an incision was made along the midline of the neck, followed by blunt dissection of muscles and connective tissues. The bilateral common carotid arteries were isolated and ligated with double silk sutures. The skin was sutured after operation. The rats were randomly assigned to the sham-operated group and the operated group. For rats of the sham-operated group, the bilateral carotid arteries were separated without ligation. Eight rats died 1 week after operation, including 5 rats in the operated group and 3 in the sham-operated group. Six weeks after operation, animals in the operated group were randomly assigned to the experimental group and the control group, with 15 rats in each group. The other 7 rats were used for replacement. The experimental group was given CIT (Lexapro, manufactured by H. Lundbeck, Denmark) at a dose of 2 mg/kg every day (prepared to 5 mL with PBS) by gavage for 14 consecutive days. The control group was treated with PBS (5 mL) by gavage for 14 consecutive days. The sham-operated group was not given any intervention during the experiment. There were no marked differences in feeding and housing conditions in all groups.

**1.2 Morris Water Maze Test**

After continuous intervention for 14 days (postoperative 8 weeks), Morris water maze test[9] was carried out. The test consisted of two periods. In the first period, the 5-day positioned navigation test was conducted, in which the rats arbitrarily accessed the water from a location in front of pool wall in northeast (NE), northwest (NW), southwest (SW) or southeast (SE) quadrants. The swim path and time to successfully enter the platform were recorded within 60 s. If the rats failed to enter the platform within 60 s, they were instructed to locate the platform and maintained at the platform for 15 s and the escape latency was recorded as 60 s. The interval between exercises was 60 s. In the second period, the spatial probe test was conducted on the last day of the experiment, in which the platform was removed and the rat accessed the water from a location in the SW quadrant. The time spent in the NE quadrant and swim path were recorded within 30 s. Seven rats that failed the Morris water maze test were excluded, including 3 rats in the experimental group, 2 in the control group, and 2 in the sham-operated group.

**1.3 Proliferation and Differentiation of BrdU-labeled Cerebral Cells**

The newly generated cells in brain tissues were detected using BrdU as per instruction provided by Sigma (USA). The brain tissues were fixed in neutral buffered formalin, dehydrated in graded ethanol, embedded in paraffin, sectioned, melted at 56°C, dewaxed in xylene and stained with HE for pathological examination. The angiogenesis of ischemic cerebral tissue was determined using immunohistochemical method with BrdU antibody as primary antibody. Five fields were selected in the same area of ischemic cortex. The number ($\bar{x}\pm s$) of BrdU positive cells in ischemic cerebral cortex was determined for each group using HPIAS 2000 software. The cells in the homogeneous area of the opposite site were regarded as control cells. The PBS was used in the blank control to substitute primary antibody.

**1.4 Confocal Three-dimensional Imaging of Cerebral Vessels**

Two weeks after intervention, the vessels of ischemic brain were examined in each group ($n$=6 per group) using confocal three-dimensional imaging of cerebral vessels according to Chen *et al*[3]. Six rats in each group were normally anesthetized and given plasma labeled with fluorescein isothiocyanate (FITC)-dextran (relative molecular mass of $2\times10^6$, 50 $mg\cdot mL^{-1}\cdot kg^{-1}$, Sigma, USA) via the femoral vein. One min later, the brain was quickly extracted from the rat cranium and placed in 4% paraformaldehyde in an ice box, and fixed for 24–48 h under protection from light. The specimen was sliced into coronal sections using vibratome. The optic chiasma was collected and serial sections at a thickness of 100 μm were prepared toward the cerebrum. 100 sections were scanned using confocal laser scanning microscope. The green fluorescent volume meant the volume of plasma perfusion. The images of vessels were obtained using a magnification of 40×. The vessels of less than 8 mm in diameter were analyzed for vascular morphology, diameter, density and fluorescent points per unit area.

**1.5 Determination of Plasma VEGF Concentration Using Enzyme Linked Immunosorbent Assay**

Two weeks after modeling, the plasma VEGF concentration was determined in each group ($n$=6 per group) using enzyme linked immunosorbent assay as per the instruction of VEGF ELISA kit (Wuhan Boster Biological Technology Co., Ltd., China). The absorbance ($A$) was determined at 450 nm using RT-2100 C microplate reader (Rayto Life And Analytical Sciences Co., Ltd., USA). VEGF concentrations of rats in each group were calculated using software.

**1.6 Expression of VEGF in Ischemic Cerebral Tissues Using Immumohistochemical Staining**

Fourteen days after intervention, 6 rats in each group were examined by immumohistochemical staining. The tissue perfusion and fixation required the following procedures: 10% chloral hydrate (300 mg/kg) was intraperitoneally injected for the induction of anesthesia. The rats were then perfused with paraformaldehyde for consecutive 30 min. The brain tissues were extracted, fixed with 4% paraformaldehyde, dehydrated and embedded in paraffin. The VEGF immunohistochemical staining was performed. The mounted section was observed under the inverted microscope and photographed. Each section was photographed for 3 fields in the same ischemic area of the cortex, hippocampal CA1 area and striatum under a magnification of 400×. The immunohistochemical images were analyzed using Image-Pro Plus 6.0. The integral $A$ ($\bar{x}\pm s$) was determined for each image.

**1.7 Statistical Analysis**

The data of each group was expressed as $\bar{x}\pm s$. The data of two specimens were compared using paired $t$ test. The data between groups were compared using one-way

analysis of variance. The statistical calculation was performed using SPSS13.0 software. Statistical significance was indicated by $P<0.05$. The results of rats in Morris water maze test were expressed as $\bar{x}\pm s_{\bar{x}}$. The other data were expressed as $\bar{x}\pm s$. The comparison between two groups was performed using $t$ test. The comparison among multiple specimens was performed using multi-factor analysis of variance. The statistical calculation was performed using SPSS13.0 software. Statistical significance was indicated by $P<0.05$.

## 2 RESULTS

### 2.1 Effect of CIT on Spatial Learning and Memory of Rats with Chronic Hypoperfusion

The results are shown in table 1.

**2.1.1 Positioned Navigation Test** The latency in the positioned navigation test was primarily designed to test spatial learning and memory of rats. The mean latency of rats in four quadrants per day in each group referred to the mean latency of rats in Morris water maze per day in each group. The two-factor analysis of variance (4×5) showed there was statistically significant difference in mean latency between groups ($P<0.05$), demonstrating that the intervention and exercise days had impacts on latency of rats in Morris water maze test. The sham group had shorter latency than the operated group at day 2, 3, 4 and 5. The rats in the experimental group had following results of latency: (44.65±7.61) s, (38.17 ±1.51) s, (34.57±3.33) s, (26.17±3.62) s, markedly shorter than in the control group, which were (52.39±7.62) s, (45.71±8.11) s, (47.39±9.31) s, (49.65±7.65) s, respectively ($P<0.01$). There was statistically significant difference ($P<0.01$), indicating that the rats in the experimental group had significantly better learning and memory than in the control group.

**Table 1 Results of Morris water maze test in each group ($\bar{x}\pm s$, s)**

| Groups | Second day | Third day | Fourth day | Fifth day | Spatial probe test | $F$ | $P$ |
|---|---|---|---|---|---|---|---|
| Sham | 40.12±6.31 | 32.14±5.92 | 29.35±4.51 | 21.27±2.19 | 37.78±5.65 | 12.861 | 0.012 |
| PBS | 52.39±7.62 | 45.71±8.11 | 47.39±9.31 | 49.65±7.65 | 14.58±3.26 | 15.637 | 0.023 |
| CIT | 44.65±7.61 | 38.17±1.51 | 34.57±3.33 | 26.17±3.62 | 28.37±3.19 | 25.365 | 0.015 |
| $F$ | 11.877 | 21.365 | 41.265 | 39.283 | 50.657 | | |
| $P$ | 0.007 | 0.005 | 0.005 | 0.002 | 0.002 | | |

**2.1.2 Spatial Probe Test** The spatial probe test was primarily designed to examine memory of the platform in rats. The time spent in the first quadrant of the original platform within 30 s was recorded for rats in each group. The mean indicated the memory of the platform in rats. The one-way analysis of variance showed the experimental group (28.37±3.19 s) spent longer time than the control group (14.58±3.26 s). The difference was statistically significant ($P<0.001$), suggesting that the rats in the experimental group had better memory of the platform than the control group.

### 2.2 Detection of Density of Cerebral Vessels in Ischemic Area

The results of confocal three-dimensional imaging revealed that the experimental group had smaller diameter of blood capillary per unit area than the control group ($P=0.047<0.05$); The density of vessels in the ischemic area was significantly greater in the experimental group than the control group as regarding with the same hemisphere ($P=0.003<0.01$); for the total surface area of vessels, new data were added to the statistical analysis of three-dimensional vessels to avoid errors produced by unpaired data analysis. The comparison of total surface area of capillaries in rats regarding the same hemisphere showed the experimental group had significant increase in total surface area of vessels as compared with the control group ($P=0.004<0.05$) (table 2, fig. 1).

**Table 2 Three-dimensional quantitative results of vessel structure ($\bar{x}\pm s$)**

| Variables | Sham | PBS | CIT | F-numbers | $P1$ | $P2$ | $P3$ |
|---|---|---|---|---|---|---|---|
| Vessel diameter (μm) | 4.37±0.31 | 3.38±0.16 | 3.12±0.24 | 29.525 | 0.031 | 0.153 | 0.047 |
| Vessel density (/0.002 $mm^3$) | 135.75±9.31 | 157.62±11.52 | 207.60±11.82 | 37.089 | 0.475 | 0.003 | 0.003 |
| The total surface area of blood vessels ($\mu m^2$/0.002 $mm^3$) | 67859±2986 | 76347±2897 | 85279±3951 | 17.326 | 0.0365 | 0.002 | 0.004 |

P1: sham *vs.* PBS; P2: sham *vs.* CIT; P3: CIT *vs.* PBS

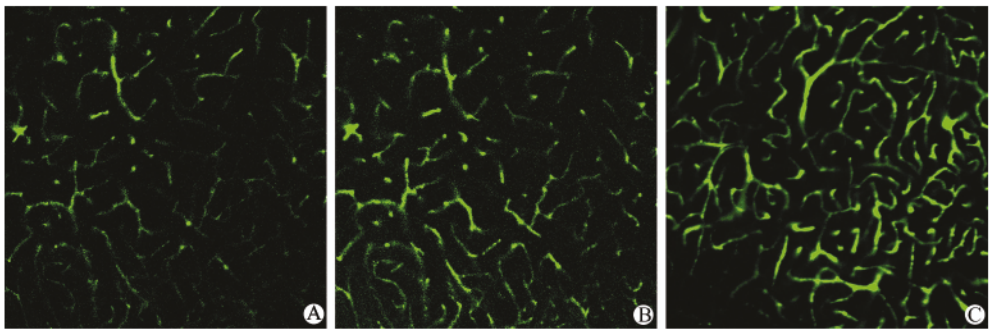


**Fig. 1** Three-dimensional confocal imaging of blood vessels of brain in each group (×400)
A: sham group; B: PBS group; C: CIT group

### 2.3 Results of Proliferation and Differentiation of Cells in Ischemic Cerebral Area

The BrdU can be incorporated into DNA chain during phase S and participate in synthesis of newly produced cells. It can be used as an effective label for newly proliferated cells. In the immunohistochemical staining, the cells stained with brown nucleus are regarded as new cells or differentiating cells. The statistical results showed the experimental group had significantly more positive stained cells (19.12±6.15) than the control group (9.27±1.68) ($P$=0.003<0.01). This indicated that the experimental group had more cells undergoing proliferation and differentiation in the ischemic cerebral tissue than the control group. The intervention effectively facilitated the cell proliferation and differentiation in the ischemic cerebral tissue.

### 2.4 Concentration of Plasma VEGF

At 14th day after operation, the concentrations of plasma VEGF were determined in the sham group, control group and experimental group using ELISA, which were 4.37±0.36, 15.65±3.52 and 39.77±3.62 pg/mL, respectively. There was significant difference among three groups ($F$=32.62, $P$=0.005<0.01). The concentrations of plasma VEGF in the control group and the experimental group were significantly higher than in the sham group ($F$=58.37, $P$=0.002<0.01), and those in the experimental group were also significantly higher than in the control group ($F$=29.69, $P$=0.003<0.01). These results showed cerebral ischemic rats had increased VEGF level after operation. However, the experimental group had significantly higher concentration of plasma VEGF, probably due to the effect of VEGF on the neuroprotection of CIT.

### 2.5 Determination of VEGF Expression in Brain Tissue Using Immumohistochemical Staining

The results of immumohistochemical staining showed VEGF positive cells had morphological characteristics of neurons, primarily present in cerebral cortex, hippocampus and corpus striatum. The cells were stained with brown in cytoplasm and expressed in neurons, spongiocytes and endothelial cells.

The $A$ values in the experimental group were significantly higher than in the sham group and the control group ($F$=47.25, $P$=0.005<0.01 for the hippocampus; $F$=78.32, $P$=0.003<0.01 for the cortex; and $F$=55.38, $P$=0.003<0.01 for the corpus striatum), further demonstrating that the expression of VEGF increased after cerebral ischemia and even higher after drug intervention (table 3).

**Table 3 Analysis of positive $A$ values in rats 2 weeks after cerebral ischemia using VEGF immunohistochemical staining ($n$=6, $\bar{x}\pm s$)**

| Groups | $A$ values | | |
|---|---|---|---|
| | Hippocampus | Cerebral cortex | Corpus striatum |
| Sham | 117.65±4.86 | 186.64±10.69 | 125.18±26.82 |
| PBS | 143.18±13.65 | 236.18±16.83 | 143.46±24.45 |
| CIT | 257.89±11.78 | 628.56±35.67 | 616.89±11.34 |
| $F$ | 47.25 | 78.32 | 55.38 |
| $P$ | 0.005 | 0.003 | 0.003 |

## 3 DISCUSSION

In the present study, the typical 2-VO model with chronic hypoperfusion was used to mimic a series of pathological and physiological processes caused by chronic hypoperfusion. At 2nd week after intervention, Morris water maze test was carried out to compare the spatial learning and memory between groups. The results showed the rats given CIT had better spatial learning and memory than those treated with PBS.

The confocal three-dimensional cerebrovascular imaging and determination of cell proliferation in the brain tissue were conducted to explore potential mechanisms. We found that the experimental group had significantly increased new cells and vessel density in the ischemic cerebral tissue, demonstrating that CIT effectively promoted the proliferation and differentiation of ischemic cerebral tissue, increased angiogenesis in the ischemic area and promoted blood supply to the ischemic cerebral tissue. At 14th day after consecutive intervention, the laser confocal observation found that the ischemic cerebral lesion had clear edge and the superficial vessel branches significantly increased. Moreover, the vessel branches were pooled in the ischemic cerebral lesion and some vessel branches travelled across the surface of the ischemic surface, indicating that CIT promoted the absorption and recovery of the ischemic lesion, and enhanced restoration of the vessel networks. The further statistical results also showed the density of new capillaries was significantly increased in the ischemic lesion of rats in the experimental group as compared with that in the control group, suggesting that intervention facilitated the angiogenesis in the ischemic area.

The angiogenesis in the brain tissue after cerebral ischemia protects the cerebral nerves and promotes recovery. The vessels are anatomically associated with nerves. They are functionally matched. The damage to the structural and functional relation between vessels and nerves will definitely cause cerebral dysfunction, which is characterized by neuronal apoptosis and necrosis in the ischemic area that worsen delayed neurological damage after cerebral ischemia. CIT promotes angiogenesis after cerebral ischemia and makes it possible to facilitate earlier recovery of blood supply around the ischemic area and reduction of ischemic cerebral injury. During recovery of nerves in rats after cerebral ischemia, the activation and shift of functional cerebral areas as well as the initiation and activation of mechanism for endogenous neural protection and recovery will increase consumption of energy in the brain. This is definitely dependent on transportation of cerebral blood flow and good perfusion in relevant cerebral areas. Hence, we speculated that an-

giogenesis is important basic anatomical structure for the CIT's role in protection after cerebral ischemia and restoration of vessel networks around the ischemic lesion.

The level of VEGF was also determined for the study of potential molecular biological mechanisms through which CIT exhibited the neuroprotection. The results of ELISA and immunohistochemical staining showed that the level of plasma VEGF was significantly higher in the experimental group than in the control group and sham group, suggesting that the CIT intervention promoted elevated expression of VEGF after cerebral ischemia. The experimental group had significantly higher *A* values than the control group and sham group, and also had significantly higher gray level of proteins than the control group, suggesting that the CIT significantly promoted expression of VEGF protein, which was also directly associated with increase in blood vessel permeability by VEGF. The results showed that CIT reduced neural damage, improved neural function and exhibited neuroprotection in cerebral ischemic rats with reperfusion, which was probably associated with angiogenesis induced by VEGF.

VEGF is a specific mitogen for endothelial cells. It is a homodimer glycoprotein with molecular weight of 45 000. It can directly act on vascular endothelial cells via flt-1 and flk-1 receptors to facilitate formation of lumen of endothelial cells. It can increase microvessel density after ischemia, improve microcirculation around ischemic lesion, promote recovery of vessel function in the late phase of reperfusion and facilitate recovery of nerves in the ischemic cerebral tissue. VEGF is the most important known angiogenic factor. It is well recognized for its neuroprotection. It can initiate angiogenesis to exhibit its biological functions such as promotion of growth of neuron[10].

Some studies had shown that VEGF as an antidepressant promotes proliferation of neural cells and increases the synaptic growth via VEGFR2 to exhibit neuroprotection, which occurs in 14–21 days[4]. The VEGFR2 has a primary role in the increase of vascular endothelial cell proliferation and expression of VEGF in the hippocampus. Some studies found[11] the VEGF-mediated cell proliferation is related to the antidepressant action with the increased expression of VEGF. Some studies found[1] that administration of SSRIs in rats after acute ischemia significantly improved cognitive function and facilitated neuronal proliferation in the hippocampus. Long-term use of SSRIs can facilitate regeneration of neurons in the hippocampus[2].

The study protocol which combined ethology with molecular biology comprehensively investigated the possible mechanisms through which CIT facilitated angiogenesis to exhibit neuroprotection. But there are still some limitations. First, although the present study found CIT facilitated angiogenesis, no further study was carried out to focus on functions such as maturity and stabilization of new vessels. Second, the possible ion channels for the CIT to facilitate angiogenesis to improve blood supply and upregulate the expression of VEGF in the brain of rats with cerebral ischemia remained to be further discussed. Third, the changes of expression in different points using different doses were not observed in the present study. Therefore, the dynamic changes of density of brain vessels and VEGF expression in ischemic area remained to be further studied.

**Conflict of Interest Statement**

No potential conflict of interest relevant to this article is reported.

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