Osthole Improves Cognitive Function of Vascular Dementia Rats: Reducing Aβ Deposition via Inhibition NLRP3 Inflammasome

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INTRODUCTION

Cognitive dysfunction is a major manifestation of vascular dementia (VD) which is a neurodegenerative disease.1) Among all dementia patients, Alzheimer’s disease (AD) ranked first, and VD second. VD currently has no effective treatment, as a result, the family and society have suffered a great economic burden, it is very urgent to develop a new drug or therapy for it.2) VD has a variety of different subtypes which exists the heterogeneity among them, but the study indicated that one of the starting conditions for VD is chronic cerebral hypoperfusion (CCH), after angiogenesis and collateral circulation are established, CCH status will be reversed.3) CCH is a chronic and long-term state in which cerebral blood flow continues to decrease, thereby impairing the function of neurons which leads to cognitive dysfunction.4) In order to imitate VD model, according to literatures the CCH rats in this study were operated the modified bilateral common carotid artery occlusion (BCCAO).5)

Currently, VD has quite complex pathogenesis, such as beta-amyloid (Aβ) deposition, tau hyperphosphorylation, inflammatory response etc.6–8) Furthermore, lots of evidences indicated that the rats induced by BCCAO emerged progressive cognitive dysfunction, Aβ deposition and increasing of inflammatory response, the process was reversed after treatment with medication.9,10) Neuroinflammation has both positive and negative sides, either too strong or too weak inflammation is harmful to neurons.11,12) The study showed that amyloid precursor protein (APP) can maintain the plasticity of neurons, conserve neurons, and then alleviate the cognitive dysfunction, a large amount of APP appears in hippocampus of rats induced by BCCAO, and APP is decomposed into Aβ by β-site APP cleavage enzyme (BACE1).13) A large number of Aβ aggregates to form Aβ deposition which will lead to microglial activation, and then over-expression of nod-like receptor protein 3 (NLRP3) inflammasome in microglial to scavenge Aβ and cause neuronal inflammatory apoptosis, and neuronal inflammatory apoptosis in turn result in over-expression of APP eventually causes the inflammatory reaction of vicious circle.14) In the Pharmacopoeia of the People’s Republic of China, there is a traditional Chinese medicine called Shechuangzi from the fruit of Cnidium monnieri (L.), and it is used to cure skin diseases and gynecological diseases, Shechuangzi has been proved to possess multiple pharmacological characteristics, including cardiovascular activity, effects on the respiratory system, neuroprotective activity, anti-anxiety and memory improvement effects, effects on the reproductive system, regulatory effects on the endocrine system, anti-inflammatory and anti-pruritic effects, immunomodulatory effect, anti-cancer effects, liver protective effect, anti-osteoporotic activity, other effects etc., so far, many different chemical components have been separated from Shechuangzi, including coumarins, volatile oils, chromones, triterpenoids, glycosides, glucides, other compounds etc.15)

Osthole (OST) is a coumarin compound from Chinese herb Shechuangzi.16) After oral administration of OST, it can be detected in the brain tissue of mouse at 5 min and 10 h later, and a certain concentration of OST can be maintained for a long time in the brain tissue of mouse, indicating that OST is absorbed rapidly and eliminated slowly in the brain tissue.17)
It has been reported that OST has a wide range of pharmacological activities, for example, anti-tumor, anti-seizure antihypertension etc.\(^{19–21}\) Numerous interesting researches indicated that OST exists neuroprotection in different animal models, for instance, improving cognitive function of AD model, preventing cerebral ischemia-reperfusion injury, enhancing adult neurogenesis by up-regulating brain derived neurotrophic factor (BDNF).\(^{19–21}\) But it is not still clear that the effect of OST on A\(\beta\) deposition and on neuroinflammation in CCH animal model. Therefore, this study aims to investigate whether OST exerts therapeutic effect on VD, and explores the possible underlying mechanisms.

**MATERIALS AND METHODS**

**Drugs** OST (\(C_{15}H_{16}O_3\), MW: 244.29, 7-methoxy-8-isopentenoxycoumarin, purity \(\geq 99.90\%\) by LC-MS) was purchased from MedChemExpress.cn (Shanghai, China) (CAS: 484-12-8), and it is mainly through chemical synthesis, tween 80 as a solvent to dissolve it.

**Animals** Sprague-Dawley (SD, adult, male) rats (260 ± 20 g) were purchased from the Changsha Tianqin Biotechnology Corporation Ltd. (Changsha, China; SPF grade, Certificate No. SCXK2014-0011). The temperature (22 ± 1°C) and humidity (55 ± 10%) are constant in the SPF-grade room, and it turns on at 8:00 and off at 20:00 every day. Per cage had five rats which were free to drink water and eat foods. The rats were raised for 2 weeks before experiment, and the animal experiments were followed the Experimental Animal Ethics Committee of Zunyi Medical University.

**Model Prepare** The rats were subjected to the modified BCCAO which followed anesthesia with 3 mL/kg intraperitoneal injections of 2% sodium pentobarbital. In short, the rats were supine fixed on the rat board, and made a 1 cm long incision about 0.3 cm to the left of the center of the neck. The left common carotid artery, vagal nerve, carotid sheath and cervical sympathetic were clearly visible between the sterno-hyoid and sternomastoid muscles. The two glass needle dividers were used to separate out the left common carotid artery which was tightly ligated with 4-0 silk sutures. Finally, the left incision was sutured with 2-0 silk sutures. After a week, the rats were subjected to the same operation on the right of the neck. The sham-operated rats were subjected the same surgery but not occlusion.

**Drug Administration and Experimental Design** The rats were randomly assigned to five groups: sham group (n = 12), BCCAO group (n = 12), BCCAO + OST 5 group (n = 12), BCCAO + OST 10 group (n = 12), and BCCAO + OST 20 group (n = 12). The treatment would be started on the 2th day after the BCCAO surgery, the rats in OST treated groups were received daily oral administration of different doses of OST 5, 10, and 20 mg/kg, respectively for 62 continuous days. Double distilled water of the same volume was given to the sham and BCCAO control groups. On the 63th day after modeling, all rats were sacrificed to prepare for subsequent experiments.

**Morris Water Maze Test** The Morris Water Maze was used to test the abilities of spatial learning and memory. The task was carried out during days 53–59 after surgery. The Morris Water Maze complete set of equipment mainly consisted of a circular black pool (diameter: 160, height: 50 cm), camera system and animal behavior trajectory analysis system. There was a platform in the pool, and the water (24 ± 2°C) was added to the submerged platform for 1 cm. We divided the pool into four quadrants: the first quadrant, the second quadrant, the third quadrant and the fourth quadrant. The platform was at the midpoint of the first quadrant. The rats were put into the water with their heads facing the pool wall, and the order was randomly selected one of the other three quadrants except the first quadrant every day. The time of finding the underwater platform was recorded as the escape latency. If the rats failed to find the platform in 120 s, the escape latency was recorded as 120 s, and then the rats were guided to the platform to rest for 15 s. The experiment lasted for 7d, there were 3 trials per animal every day, and the TopScan-Topview Behavior Analyzing System (TopScan Version 3.00) recorded the escape latency.

**Y-maze Test** The Y-maze tested the ability of spatial working memory. There were A, B, and C three identical arms at 120° in the Y-maze (length: 50, width: 10, height: 20 cm). We gently lowered the rat’s head toward the end of an arm, keeping the whole process quiet and letting it move freely in the Y-maze for 10 min. A successful spontaneous reaction alternation behavior is that the head and limbs of the rat completely enter the arm, and they must successively enter and exit the three different arms. After a test, we sprayed the device with 10% alcohol, scrubbed the device evenly with an absorbent cloth, and finally dried the device with dry toilet paper in the experiment. “\(N\)” represented the total number of arm entries, and “\(n\)” represented the sequence of entries. The spontaneous alternation rate (\(\% = \frac{n}{N(N-2)} \times 100\)) represented “\(N\)” and “\(n\)”.

**Hematoxylin–Eosin (H&E) Staining, Nissl Staining and Immunofluorescence Staining** After the Y-maze test, four rats in each group were anesthetized with 3 mL/kg intraperitoneal injections of 2% sodium pentobarbital. 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde solution were prepared in this study and stored in 4°C. Four rats were perfused transcardially with 200 mL PBS and paraformaldehyde solution respectively each group, and then the brain was immediately separated on ice plate. The brain was fixed in the 4% paraformaldehyde solution for 48 h. Finally, it went through a series of processes: dehydration, paraffin embedding and sectioning for H&E staining, Nissl staining and Immunofluorescence staining (IBA1, 1:200, Abacm, ab178847, U.K.; IBA1, 1:200, Abacm, ab15690; NLRP3, 1:200, Abacm, ab214185). The upright metallurgical microscope (BX43 + DP2b, Olympus, Japan) would be observed the damage of neurons in CA3 region of hippocampus, the positive fluorescent microscope (MBX53, Olympus) would be observed the number of microglia activated in CA3 region of hippocampus.

**Western Blot** After the Y-maze test, the rest rats in each group were sacrificed after anesthetized with 3 mL/kg intraperitoneal injections of 2% sodium pentobarbital, and the brains were collected on ice plate to prepare hippocampal tissue homogenate. Main steps: the extraction and quantification of proteins, electrophoresis, electro-transformation, blocking with 5% non-fat milk, incubation with primary antibodies with rotation at 4°C, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, finally

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reacted with enhanced chemiluminescence (ECL). The primary antibodies used in this study included the following: APP (1:1000, Abcam, ab32136), BACE1 (1:1000, Abcam, ab108394), Aβ1–40 (1:2000, Abnova, MAB2675, U.S.A.), Aβ1–42 (1:1000, Abcam, ab201060), NLRP3 (1:500, Abcam, ab214185), β-actin (1:2000, Proteintech, 66009-1-lg, China). The secondary antibodies used in this study included the following: Goat Anti-Mouse immunoglobulin G (IgG) (H+L) (1:5000, Proteintech, SA00001-1) and Goat Anti-Rabbit IgG (H+L) (1:5000, Proteintech, SA00001-2).

**Statistical Analysis** SPSS 18.0 Software was used for statistical analysis of this study, mean ± standard error of the mean (S.E.M.) as the expression of data. The experimental results of this study were analyzed by a one-way ANOVA and Bonferroni multiple comparisons test, except the escape latency which was analyzed by repeated measures ANOVA and Bonferroni multiple comparison test. *p < 0.05 was considered statistically significant.

![Fig. 1. The Chemical Structure of OST](image)

![Fig. 2. Effect of OST on Spatial Learning, Memory and Working Deficit of VD Rat](image)

(A) Experimental design: After BCCAO surgery in rats, oral administration of OST 5, 10, and 20 mg/kg to rats for 61 d. Morris Water Maze test was carried out from day 53 to day 59 after surgery for 1 week. From day 60 to day 61 after surgery, Y-maze test was performed for 2 d. (B) From day 1 to day 7, the results of the escape latency of rats in each group. (C) The average spontaneous alternation rate of rats in each group. Data were expressed as mean ± S.E.M. (n = 12). *p < 0.05, **p < 0.001 vs. sham, *p < 0.05, ***p < 0.001 vs. BCCAO.
RESULTS

**OST Attenuated Spatial Learning, Memory and Working Deficits in VD Rats** To investigate whether OST could have an effect on the behavior function, Morris Water Maze test and Y maze test were used in this study. From the day 53 after the BCCAO surgery, the spatial learning and memory abilities were observed by Morris Water Maze test lasted for 1 week (Fig. 2A). The escape latency of each group of rats was significantly shortened followed training. From day 4 to day 7, the escape latency of the BCCAO model rats was significantly longer than that of the sham rats (day 4: $p<0.05$, day 5: $p<0.001$, day 6: $p<0.001$, day 7: $p<0.001$), however, rats treated with OST in 5, 10mg/kg and 20mg/kg groups significantly shortened the escape latency than BCCAO rats ($p<0.05$, $p<0.01$ and $p<0.001$ in Fig. 2B).

The spontaneous alternation behavior was used to evaluate the spatial working memory of the rats in the Y-maze test. From the day 60 after surgery, we performed the 2-d Y-maze test (Fig. 2A) to investigate whether OST could attenuate spatial working memory deficit which was induced by BCCAO. The results showed that the spontaneous alter-
nation rate of BCCAO rats was signally lower than that of the sham group \((p<0.001)\), however, rats treated with OST in 5 mg/kg \((p<0.05)\), 10 mg/kg \((p<0.001)\), and 20 mg/kg \((p<0.05)\) groups were observably increased the spontaneous alternation rate compared with BCCAO rats (Fig. 2C).

**OST Alleviated the Damage of Neurons in the Hippocampus of VD Rats**  
H&E and Nissl staining were used to examine the damage of neurons in CA3 region of hippocampus induced by BCCAO. Compared with the sham group, the neurons were obviously pathological damage in CA3 region of BCCAO rats by H&E stained, however, the neurons damage was distinctly alleviated in rats treated with OST (5, 10, and 20 mg/kg, respectively) (Fig. 3).

Compared with the sham group, few Nissl bodies and intact cells were found in hippocampal CA3 region of BCCAO rats by Nissl stained \((p<0.001)\), followed OST treatment (5 mg/kg \((p<0.001)\), 10 mg/kg \((p<0.001)\), 20 mg/kg \((p<0.001)\)), the number of Nissl bodies and intact cells were visibly increased (Fig. 4). The results of H&E and Nissl staining showed that OST alleviated the damage of neurons in the hippocampus of VD rats.

**OST Inhibited the Activation of Microglia in the Hippocampus of VD Rats**  
Immunofluorescence staining was used to observe the number of microglia activated in CA3 region of hippocampus induced by BCCAO. Compared with the sham group, the number of microglia activated were obviously increased in hippocampal CA3 region of BCCAO rats \((p<0.001)\), followed OST treatment (5 mg/kg \((p<0.001)\), 10 mg/kg \((p<0.001)\), 20 mg/kg \((p<0.001)\)), the number of microglia activated was visibly reduced. The result of Immunofluorescence staining indicated that OST inhibited the activation of microglia in the hippocampus of VD rats (Fig. 5).

**OST Inhibited Microglial Activation and NLRP3 Protein Expression in the Hippocampus of VD Rats**  
In order to explore the relationship of activated microglial activation and NLRP3 in the hippocampus of VD rats, the number of microglial activation and NLRP3 protein expression were examined by double immunofluorescence staining with anti-IBA1 and anti-NLRP3 antibodies. Compared with the sham group, the number of microglial activation and NLRP3 protein expression were obviously increased in the hippocampus of VD rats \((IBA1: p<0.001; NLRP3: p<0.001)\), and treatment with OST \((5 \text{ mg/kg (IBA1: } p<0.001, \ NLRP3: p<0.001), 10 \text{ mg/kg (IBA1: } p<0.001, \ NLRP3: p<0.001), 20 \text{ mg/kg (IBA1: } p<0.001, \ NLRP3: p<0.001)}\), respectively} evidently reduced the number of microglial activation and NLRP3 pro-
tein expression compared with BCCAO rats (Fig. 6).

**OST Down-Regulated the Level of NLRP3 in the Hippocampus of VD Rats** Compared with the sham group, the expression of NLRP3 of hippocampus was obviously increased in the BCCAO model group by Western blot test \((p < 0.001)\), and treatment with OST \(5 \text{ mg/kg} (p < 0.001), 10 \text{ mg/kg} (p < 0.001), 20 \text{ mg/kg} (p < 0.001)\), respectively) decreased the level of NLRP3 expression compared with the BCCAO model group (Fig. 7). These findings showed that OST could attenuate NLRP3 expression in the hippocampus of VD rats.

**OST Reduced the Levels of \(A\beta_{1-40}\) and \(A\beta_{1-42}\) Oligomers Deposited in the Hippocampus of VD Rats** Compared with the sham group, the deposition of \(A\beta_{1-40}\) and \(A\beta_{1-42}\) oligomers in hippocampus of BCCAO rats were obviously increased in Western blot \((A\beta_{1-40}: p < 0.01, A\beta_{1-42}: p < 0.05)\), and treatment with OST \(5 \text{ mg/kg} (A\beta_{1-40}: p < 0.05, A\beta_{1-42}: p < 0.01), 10 \text{ mg/kg} (A\beta_{1-40}: p < 0.001, A\beta_{1-42}: p < 0.01), 20 \text{ mg/kg} (A\beta_{1-40}: p < 0.001, A\beta_{1-42}: p < 0.01)\), respectively) markedly decreased the levels of \(A\beta_{1-40}\) and \(A\beta_{1-42}\) oligomers in the VD rat’s hippocampus.

**OST Decreased the Levels of APP and BACE1 in the Hippocampus of VD Rats** In order to study the mechanism of OST inhibiting the increase of \(A\beta\), Western blot detected the protein expression levels of APP and BACE1. Compared with the sham rats, the expression of APP and BACE1 in hippocampus were overtly increased in BCCAO rats (APP: \(p < 0.001\); BACE1: \(p < 0.001\)), and treatment with OST \(5 \text{ mg/kg} (\text{APP: } p < 0.01, \text{BACE1: } p < 0.01), 10 \text{ mg/kg} (\text{APP: } p < 0.001, \text{BACE1: } p < 0.001), 20 \text{ mg/kg} (\text{APP: } p < 0.001, \text{BACE1: } p < 0.001)\), respectively) distinctly reduced the levels of APP and BACE1 expression compared with BCCAO rats (Fig. 9).
DISCUSSION

This study verified that OST attenuated cognitive dysfunction in VD rats induced by BCCAO, evidenced by attenuating spatial learning, memory and working deficits, alleviating the neurons damage and reducing the number of microglia activated in hippocampal CA3 region, decreasing the deposition of $\beta\text{A}_{1-40}$, $\beta\text{A}_{1-42}$ oligomers in hippocampus, and down-regulating the expressions of APP, BACE1, NLRP3 proteins in the VD rats’ hippocampus. Together with these results in the study indicated that the effect of OST on VD rats is related to the regulation of APP-$\beta\text{A}$ pathway and inhibition inflamma-some in the hippocampus.

The previous studies discovered that rats exhibited a remarkable decrease of cognitive function induced by BCCAO.22) The results from Y-maze and Morris Water Maze demonstrated that the prominent spatial learning, memory and working deficits appeared in BCCAO rats of this study. Nevertheless, OST remarkably reversed the cognitive dysfunction after chronic treatment. Moreover, H&E and Nissl staining disclosed that the neurons damage of hippocampal CA3 area was partially reversed by OST treatment, which was accord-ance to the behavioral results. This study strongly proved that OST possesses the therapeutic effect on BCCAO induced-cognitive dysfunction.

APP, glycosylated receptor proteins located on the surface...
of cell membranes, is made up of 695 amino acid fragments, as a crucial precursor protein. It can maintain the plasticity of neurons and conserve neurons, it has two decomposition pathways: amyloidogenic pathway and non-amyloidogenic pathway.\(^{23}\) During the early processing of APP, BACE1 is a membrane-bound aspartic protease which is a vital enzyme.\(^{24}\) In the amyloidogenic pathway, APP is decomposed into sAPP\(\alpha\) and C99 by BACE1 at N-terminal of A\(\beta\) area, and C99 is further decomposed by \(\gamma\)-secretase to produce a large amount of A\(\beta_{1-40}\) and a small amount of A\(\beta_{1-42}\) with neurotoxicity.\(^{25}\) On the other hand, in the non-amyloidogenic pathway, APP is decomposed into neuroprotective soluble amyloid precursor protein\(\alpha\) and C83 by \(\alpha\)-secretase at N-terminal of A\(\beta\) area, and C83 is further decomposed into P3 by \(\gamma\)-secretase, which avoids the generation of A\(\beta_{1-42}\). A\(\beta\) peptide has a \(\beta\)-lamella secondary structure of polypeptide, and it is made up of 39–43 amino acid fragments.\(^{27}\) A\(\beta\) is normal product in the brain, the amount of physical A\(\beta\) can have the inhibition effect of the over-activation neurons, and it is greatly necessary to keep neurons functioning properly and continuously. A\(\beta_{1-40}\) and A\(\beta_{1-42}\) are major components of amyloid plaques, and its' oligomers exhibited the strongest neurotoxicity.\(^{28}\) Numerous evidences indicated that the imbalance between A\(\beta\) production and clearance in the brain leads to neurodegenerative diseases, such as AD and VD.\(^{29}\) There are many reasons for the increasing A\(\beta\), it is reported that the neuroinflammation plays a predominant role in A\(\beta\) imbalance.\(^{30}\) In this study, rats induced modified BCCAO showed that APP, BACE1, A\(\beta_{1-40}\), A\(\beta_{1-42}\) protein were increased, and microglial activation accompanied with the increase of NLRP3 protein were due to inflammation, however the increase of A\(\beta\) would promote inflammatory response in turn ultimately resulted in a vicious infinite circle.

In conclusion, this study elucidated that OST could obviously enhance learning and memory function and the ability of spatial working memory, improve the pathological damage CA3 area of hippocampus. Furthermore, this study suggested that OST could reduce A\(\beta\) deposition via inhibition over-expression of NLRP3 in microglial to produce anti-neuroinflammatory response in VD.

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Conflict of Interest The authors declare no conflict of interest.

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