Protective Effects of Breviscapine on Ischemic Vascular Dementia in Rats

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Breviscapine, a traditional Chinese medicine, is extensively used in clinic to treat cardiovascular diseases and cerebrovascular injury. In this study, we demonstrated the effects of breviscapine on vascular dementia (VD) rats, which were mimicked by permanent occlusion of bilateral common carotid arteries. Breviscapine (2 mg/kg for 14 d) improved the performance of learning and memory of VD rats in Morris water maze, decreased the level of lipid peroxidation and free radicals, and attenuated the pathological alterations, such as nuclear shrink, cellular edema and irregular arrangement of pyramidal layer in the hippocampal CA_1 area. *In vitro* experiment, breviscapine (50 μg/l) protected cortical neuron from injury and decreased intracellular calcium overloading induced by H_2O_2 (10 mM). The results suggest that breviscapine has therapeutic effect on cerebral ischemia and vascular dementia.

Key words breviscapine; vascular dementia; learning; memory; neuroprotection

With an increase of elderly population, aging-related diseases such as hypertension, arteriosclerosis and different forms of dementia are also increased.[1)] One of these diseases is vascular dementia (VD). VD is a step-wise deterioration in intellectual powers that appear as different areas of the brain are damaged by loss of blood supply.[2,3)] The conventional concept of VD is that of multi-infarct dementia,[3,4)] arising from chronic cerebral ischemia and various vascular changes in the brain.[5)] It is reported that the reduction in cerebral blood flow and abnormal energy metabolism occurred in chronic cerebral ischemia can induce the accumulation of NO and ROS which lead to neurons injury in selective, vulnerable regions of the brain, especially the hippocampus and cerebral cortex, accompanied by cognitive decline and some motor disorders.[6—8)]

In oriental medicine, many traditional Chinese drugs have been used clinically for treatment of ischemic cerebrovascular disease.[9)] Breviscapine is extracted from the traditional Chinese drug *Erigeron Breviscapus* (vant.) Hand.-Mazz and mainly composed of Scutellarein, Plantaginin and Pyromeconic acid.[10,11)] The injection preparation of breviscapine has been used in clinic to treat cardiovascular diseases, cerebral infarction and stroke in China.[3,10,11)] The clinical reports suggested that the injection of breviscapine (0.5 mg/kg, i.v. gtt, qd, 15 d) possess neuroprotective action and improves learning and memory in cerebrovascular diseases.[12,13)] Although the curative effect of breviscapine in cerebral infarction and cerebral ischemia is obvious, there is no data available for this drug in treatment of VD. In this study, the effects of chronic treatment with breviscapine on the learning deficits, neuronal injury and antioxidative properties in VD rats were investigated.

MATERIALS AND METHODS

Animal Models and Drug Administration The vascular dementia models were made from the male Wistar rats (6 weeks, 230±10 g), which were obtained from the Experimental Animals Center of Tongji Medical College, Huazhong University of Science and Technology. Wistar rats were subjected to permanent occlusion of bilateral common carotid arteries to induce chronic cerebral ischemia and mimic the VD models.[14)] The experimental protocols were approved by the Committee of Animal Care of Huazhong University of Science and Technology.

The injection of breviscapine (Bre, Bath No. z5302066, China) was purchased from Kunming Long-Jin Pharmaceutical Co., Ltd. Tacrine (9-amino-1,2,3,4-tetrahydroacridine HCl), a reference drug for behavioral test, was purchased from Sigma (St. Louis, MO, U.S.A.) and dissolved in physiological saline. Fifteen days after surgery, the male Wistar rats were randomly divided into three groups, *e.g.*, sham-operated group, model group, Bre-treated group and tacrine-treated group. The Bre-treated group was administered with breviscapine (2 mg/kg, i.p., equal 0.33 mg/kg adult dose, once a day). Tacrine was administered intraperitoneally at a dosage of 3 mg/kg. The sham-operated group and the model group were injected with the same volume of saline. During the behavior tests, drugs or saline were administered 30 min before the trials. The process of administering drugs lasted for 14 d.

Behavioral Test The spatial learning performance of rat was evaluated by Morris water maze.[15,16)] The experiment protocols were described in our previous study.[17)] For descriptive data collection, the pool was divided in four zones by the software (Ethovision 2.0, Noldus, Wagenigen, Netherlands). The hidden platform was placed in one of the zones. Each rat received four trails everyday and the test lasted for 5 d. The escape latency (time to reach the platform) was generally used to assess performance of learning and memory of the rats. If the rat failed to find the hidden platform within 120 s, we placed the rat on the platform. In our experiment, we mainly selected the performance in the zone where the rat released at the starting position farthest from the hidden platform. The escape latency on the fifth day of swimming training was adopted to evaluate the ability of learning and memory in rats.

The retention of spatial memory was assessed on the sixth day of swimming training by the spatial probe test. After the

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end of place navigation test, the hidden platform was removed and each rat was placed at a starting position in the pool at random. Each rat swam for 60 s. the swimming trace and distance in the target zone where the platform had been set were used to evaluate the cognitive performance. In order to determine whether the group difference in escape latency and swimming distance were due to their difference in swimming ability, the swimming speed was also evaluated.

Biochemical Analysis The sham-operated group, model group and Bre-treated group were decapitated immediately after the last behavioral test. The brains were taken out for measuring the levels of superoxide dismutase (SOD) and malondialdehyde (MDA). The content of MDA was commonly determined by the modified thiobarbituric acid (TBA) method.[18] SOD activity was detected according to the report of Ohkawa *et al.*[19] The activity of one unit SOD was defined as the enzyme amount causing 50% inhibition in the $NBTH_2$ reduction rate. SOD activity was also expressed as U/mg protein of tissue sediment. Based on these methods, the SOD activity and MDA level were measured according to the reagent kit instruction (Nanjing Jian-cheng Institute of Biological Engineering, China).

Histopathological Observation At the end of behavioral test, the rats deeply anaesthetized with chloral hydrate (350 mg/kg, i.p.), and then perfused transcardially with 100 ml saline followed by phosphate buffer (0.1 mol/l PB, pH 7.4) of 4% paraformaldehyde 400 ml. Then paraffin-embedded, coronal and serial sections (4 μm thick) were taken from each brain.[20] The sections selected at the same layers from each rat were stained with hematoxylin and eosin and evaluated using a light microscope ($\times$400) by an examiner blinded to experimental conditions. Neurons with a round- or oval-shaped nuclei and without shrinkage or edema were scored as undamaged. The number of living neurons per 0.1 mm field in the middle CA1 of the hippocampus was counted. Cell counts were made in five different fields and the numbers were averaged to yield single values for each rat.

Cell Culture Primary cortical neurons were prepared from Wistar neonate rats (within 3 d).[21,22] Briefly, under sterile conditions, cortical neurons were isolated and plated in ice-cold PBS balanced salt solution containing (in mM): NaCl 136.89, KCl 2.68, Na_2HPO_4 9.75, KH_2PO_4 1.15, pH 7.2. The tissues were mechanically dissected by eye scissors and placed into PBS containing 0.125% trypsin. The tissues were dissociated for 28 min. Dissociated cells were cultured in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37 °C (Shellab 2306, U.S.A.). The cortical neurons were plated in 96-well culture dishes or in 35 mm dishes, which were all coated with 0.01% poly-D-lysine (Sigma, U.S.A.). On the second day after seeding, the culture media were added with cytosine to inhibit the proliferation of glial elements. Cortical neurons were cultured for 6 d before drug treatment.

Measurement of Cell Viability Neuronal survival analysis was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction activity assay to access mitochondrial activity. For MTT reduction activity assay, cells had been incubated in culture medium with 5% serum before being treated with H_2O_2 (5$\sim$500 μM, Sigma) for 6 h. To observe the protective effect of breviscapine, the cells were pretreated with breviscapine (6.25$\sim$100 μg/l) for 24 h and then exposed to H_2O_2 (150 μM, Sigma) for 6 h. After that, cells were treated with MTT solution (0.5 mg/ml, Sigma) for 4 h. The dark blue formazan crystals formed in intact cells were solved with DMSO (Sigma) and shaken for 10 min. The absorbance at 490 nm was measured with a microplate reader (ELX800, Bio-Tek Instruments Inc., Coleparmar, U.S.A.). Cultured medium with 5% serum was used as blank solution. The percentage of absorbance was used to assess cell viability, assuming that the absorbance of control cell was 100%.

Calcium Imaging Measurement The calcium imaging measurement technique was described in our previous study.[23] Briefly, the cortical neurons were loaded 1 μM fura-2 in extracellular solution containing (in mM): NaCl 145, KCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 2, glucose 10, HEPES 10, pH 7.2 for 25 min at 37 °C, then washed twice to remove remaining fura-2. Fura-2 was excited by a xenon light source at 340 and 380 nm. The emitted fluorescence was filtered through a 520 nm filter, captured with an intensified CCD camera and analyzed with Tillvision software (Till Photonics, Germany). The ratio of the fluorescence (F340/F380) was used to assess intracellular calcium concentration.

Data Analysis The results were expressed as mean$\pm$S.E.M. Dunnett-*t* of analysis of variance was used to evaluate the significance of the experimental data. Statistical significance was accepted at the conventional $p<0.05$ level.

RESULTS

Breviscapine Improved the Learning and Memory of VD Rats The learning and memory ability was tested in Morris water maze. As shown in Fig. 1A, the escape latency in sham-operated group and drug-treated groups (Bre-treated group and tacrine-treated group) were markedly shorter than that in model group. The rats in sham-operated group spent 13.87$\pm$6.31 s (n=11) in finding the platform. Compared with the sham-operated group, the escape latency was significantly prolonged in model group (44.25$\pm$11.04 s, $p<0.01$, n=9). Compared with the model group, the prolonged latency was significantly reduced in Bre-treated group (15.12$\pm$2.75 s, $p<0.01$, n=9) which was similar to that of tacrine-treated group (15.06$\pm$5.01 s, $p<0.01$, n=9). Figure 1B shows the swimming traces of the model group in the spatial probe test uniformly distributed around four zones. However, the swimming traces of sham-operated and drug-treated groups concentrated in the target zone where the platform had been set. The results in Fig. 1C were parallel to Fig. 1B. Rats in the sham-operated and drug-treated groups remained with a longer swimming distance in the target zone than model group rats ($p<0.05$). Figure 1D shows that the analysis of swimming speed did not reveal any significant differences between each group ($p>0.05$).

Effects of Breviscapine on the Activity of SOD and Content of MDA Table 1 shows the activity of SOD and content of MDA in cerebral cortex and hippocampus. In model group, the activity of SOD was decreased significantly ($p<0.05$, n=8), whereas MDA content was increased ($p<0.05$, n=8) compared with that in sham-operated group. However, the decrease of the activity of SOD and the increase of the level of MDA were significantly corrected by

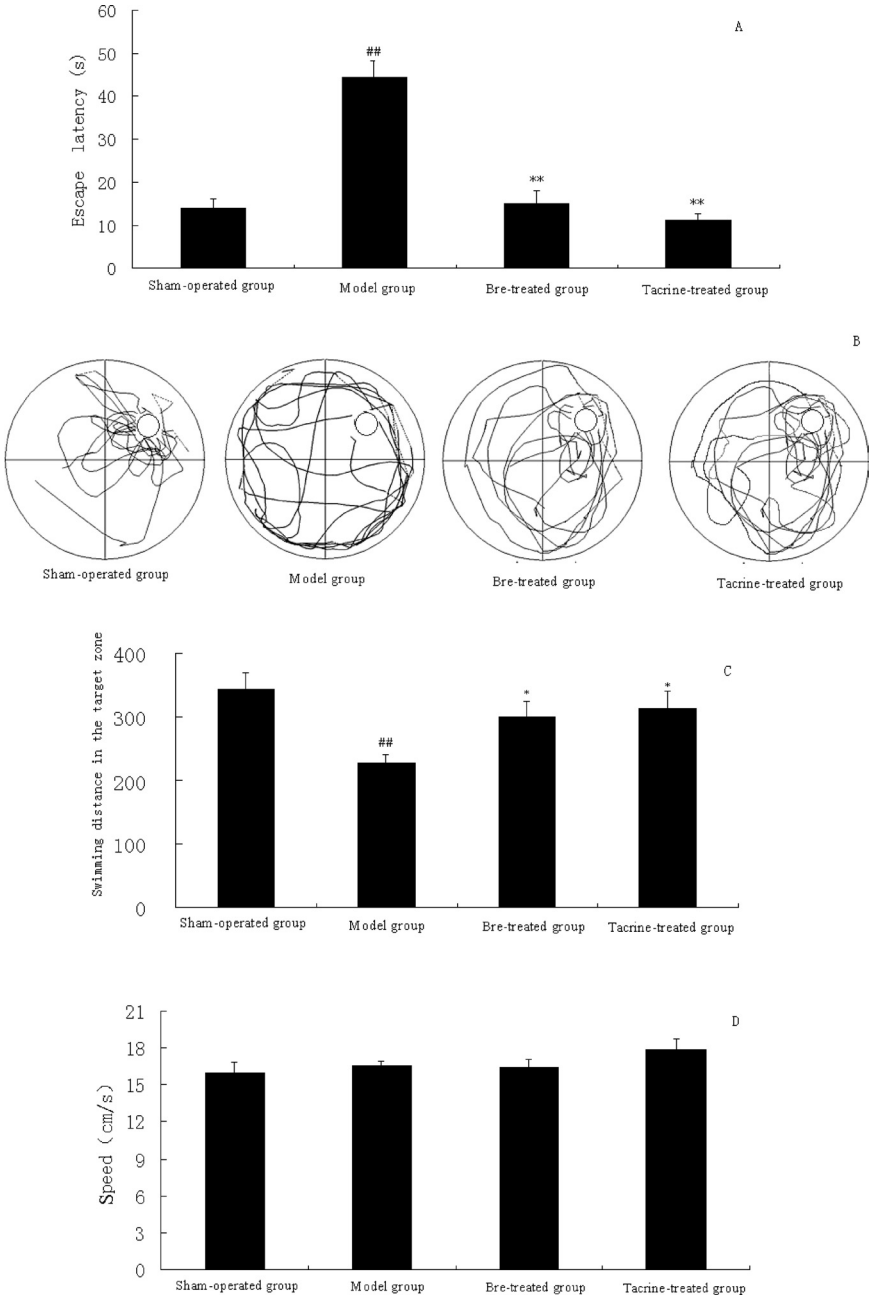

Fig. 1. Effect of Breviscapine on the Morris Water Maze Performance of the Rats (n=9—11)

(A) Escape latency to find the hidden platform. (B) Swimming trace in the spatial probe test. (C) Swimming distance in the target zone in the spatial probe test (unit: cm). (D) Swimming speed in the spatial probe test. $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with sham-operated group. $^{*}p<0.05$, $^{**}p<0.01$, compared with model group.

Table 1. Effects of Breviscapine on the Levels of MDA and the Activities of SOD in Cerebral Cortex and Hippocampus of the Rats

Group	n	Cerebral cortex		Hippocampus	
		MDA (nmol/mgpr)	SOD (NU/mgpr)	MDA (nmol/mgpr)	SOD (NU/mgpr)
Sham-operated	8	16.73±6.44	465.55±14.78	27.54±9.02	187.11±17.93
Model	8	31.41±4.8$^{\#\#}$	323.67±20.32$^{\#}$	53.79±18.63$^{\#}$	152.37±16.47$^{\#}$
Bre-treated	8	17.59±4.75**	410.13±31.61*	20.88±7.88**	195.12±12.68*

Data were denoted as mean±S.E.M. $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with sham-operated group. $^{*}p<0.05$, $^{**}p<0.01$, compared with model group.

breviscapine ($p<0.05$, $n=8$).

Effects of Breviscapine on Morphology of Hippocampal CA_1 Area Figure 2 illustrates that breviscapine prevented the histological injury induced by chronic cerebral ischemia in rat brains. In model group, permanent occlusion of bilateral common carotid arteries caused histological appearance of neuron injury in hippocampal CA_1 area. Pyramidal cellular nuclear was shrinked and accompanied by cellular

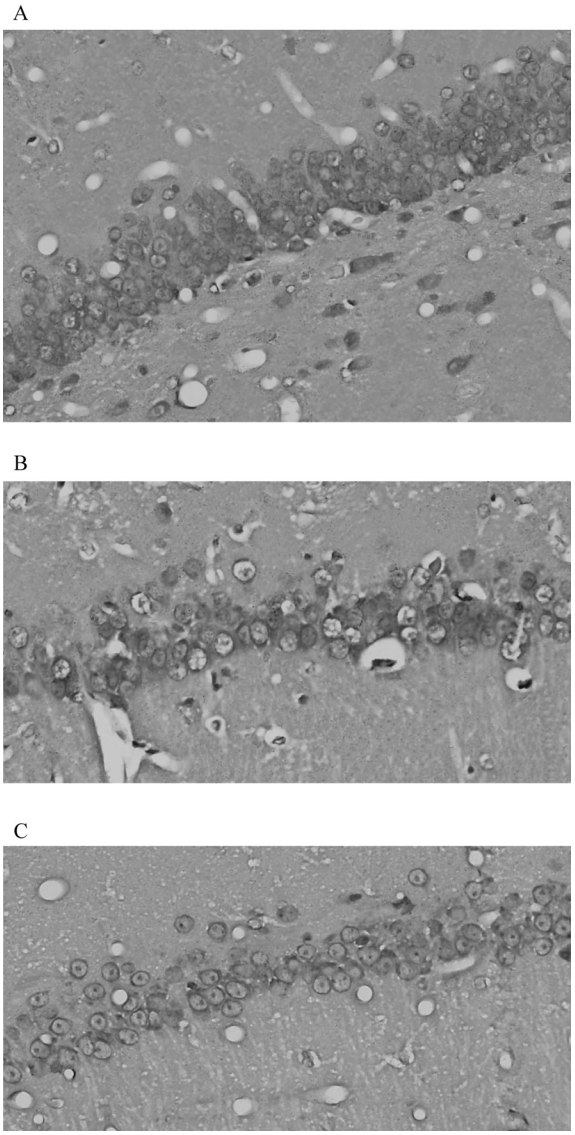

Fig. 2. Representatives of Histopathological Changes (HE Staining) in Pyramidal Layer of Hippocampal CA1 Area under a Light Microscope (×400) (n=6)

(A) Sham-operated group. (B) Model group. (C) Bre-treated group.

edema. Furthermore, the pyramidal layer was disordered and irregular in the arrangement. In contrast, the abnormal morphology in hippocampal CA_1 area was significantly relieved in Bre-treated group. In addition, cell counting showed that the number of neurons of hippocampal CA_1 area in the model group rats was decreased significantly. Compared with the sham-operated group, the number of neurons was decrease to 81.84±3.89%/0.1 mm (p<0.05, n=6) in the model group. The decrease was reduced to 92.80±3.74%/0.1 mm in the Bre-treated group (p<0.05, n=6 *vs.* the model group).

Neuroprotective Effect of Breviscapine on H_2O_2 Induced Cell Injury Cell injury model was induced by different concentration of H_2O_2. As shown in Fig. 3A, H_2O_2 decreased the cellular viability in a concentration-dependent manner. Pretreatment of breviscapine significantly attenuated H_2O_2 (150 μM)-induced cytotoxic effects. The best concentration of breviscapine for the protection was 50 μg/l, at which the cellular viability was 76.51±18.79% (n=24) and

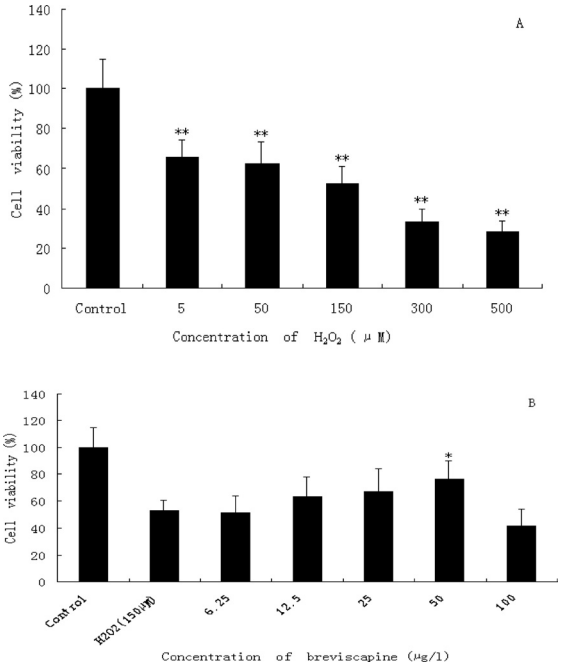

Fig. 3. Neuroprotective Effect of Breviscapine on H_2O_2 Induced Cell Injury (n=24)

(A) H_2O_2 induced cytotioxicity in a dose-dependent manner. (B) Breviscapine attenuated the cytotoxicity induced by H_2O_2. Each column and bar represented mean±S.E.M. $*p$<0.05, compared with H_2O_2 group, $**p$<0.01, compared with control group.

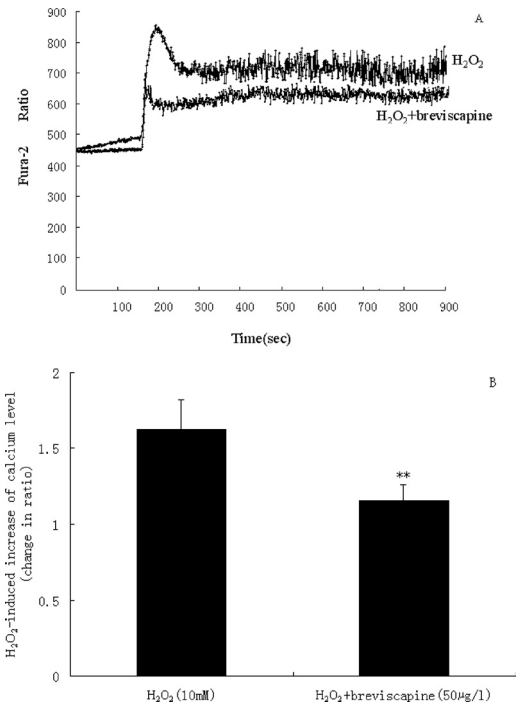

Fig. 4. Intracellular Calcium Concentration in Responses to H_2O_2 in the Presence or Absence of Breviscapine in Cultured Cortical Neurons (n=13)

(A) Each trace represents $F340/F380$ ratio from a representative cortical neuron. (B) The group data shows that breviscapine decreased the intracellular calcium elevation. $**p$<0.01, compared with H_2O_2-induced group.

was much higher than that in the model group (p<0.05, n=24, Fig. 3B).

Breviscapine Decreased the Elevation of Intracellular Calcium by H_2O_2 As shown in Fig. 4, H_2O_2 at the concen-

tration of 10 mM induced a significant increase of intracellular calcium. On the contrary, pretreatment of breviscapine 50 μg/l for 24 h prevented the elevation of intracellular calcium induced by H_2O_2. The intracellular calcium concentration could be significantly decreased by 29.10±0.08% relative to control ($p<0.01$, $n=13$) after pretreatment of breviscapine.

DISCUSSION

In the present study, we found that breviscapine improved the symptoms of vascular dementia in rats and protected the neurons from free radicals-induced injury and calcium elevation. Vascular dementia remains the second common diseases among age-related dementia.[24,25] and it is usually thought to be caused by various cerebrovascular injury.[26—28] Many studies have demonstrated that cerebral ischemia can develop cognitive deficit and neuronal damage.[6,29] According to clinical observation, the patients of VD are generally accompanied with cerebral ischemia and cognitive deficit.[29—31] Therefore, we selected the animal model of permanent occlusion of bilateral common carotid arteries to mimic the VD model. The pathogenesis of VD is very complex and still not clear completely. The symptoms of VD may correlate with the injury of cerebral cortex and hippocampus induced by the increase of free radicals.[24,32,33] Oxidative stress caused by the increase of intracellular reactive oxygen species (ROS) has been proven in relation to degenerative disorders, including premature aging, Alzheimer's disease, vascular dementia.[34,35] When cerebral neurons were attacked by excessive free radicals, the content of MDA and the activity of SOD can be increased and decreased, respectively. SOD can scavenge excessive free radicals and protect neuronal damage from free radicals.[28,36] MDA, the by-product of lipid peroxidation, can result in protein hinge and the disruption of protein synthesis, leading to obvious cognitive deficit.[36,37] In our present study, the biochemical analysis results also indicated that the content of MDA was increased and activity of SOD was decreased, which ran parallel with the cognitive impairment and histopathological changes. On the other hand, the close link between vascular dementia and Alzheimer's disease is gradually being discovered.[38] Previous studies have demonstrated that central cholinergic system is damaged by permanent 2VO rats[39—41] and stimulation of central cholinergic systems improves spatial memory deficits in 2VO rats.[42] So we selected tacrine as a reference drug in the behavior test.

Breviscapine is a well-known traditional Chinese medicine and its injection has long been used to treat cerebral ischemia.[9] Clinic observations indicate that breviscapine can ameliorate ischemia-induced cognitive impairment and cerebral infarction.[12,13,43] The curative dosage of breviscapine (0.5 mg/kg, i.v. gtt, qd, 15 d) in clinic is approximately equal to the dosage (2 mg/kg, 14 d) used in the present study. In the behavioral test, we found that the learning performance of VD rats was significantly improved by breviscapine, indicating that breviscapine has potential therapeutic value. Coincidently, it is supported by a recent study,[44] in which scutellaria flavoneid, a component of breviscapine, improved the cognitive impairment induce by permanent global ischemia. On the histopathological observation, cellular nuclear shrinkage, cellular edema, and irregular arrangement of pyramidal layer were markedly attenuated in Bre-treated group. Moreover, breviscapine decreased the content of MDA and elevated the activity of SOD. These findings are consistent with clinic observations as well as previous studies.[12,44,45] Therefore, the present study *in vivo* further demonstrates that breviscapine may attenuate chronic cerebral ischemia-induced learning and memory impairment and histopathological injury in brain tissues through decreasing content of MDA and increasing activity of SOD, which is beneficial to treat VD.

In vitro experiment, we selected the model of cortical neuron damage induced by H_2O_2 in order to investigate oxidative stress action on VD. As we known, oxidative stress easily produces lipid peroxidation resulting in the increase of cell death.[21,32] H_2O_2, one of the major component of ROS, can directly stimulate endonuclease activity in cells, leading to DNA fragmentation and cell death.[20,46] In MTT test, breviscapine relieved the cell toxicity induced by H_2O_2 in a dose-dependant manner. The viability of cells in Bre-treated group is significantly raised, suggesting that breviscapine may relieve cell toxiciy through decreasing the amount of ROS. The exposure of cortical neuron to H_2O_2 can increase intracellular calcium concentration. Given that the intracellular calcium overloading also contribute to cell toxicity and cell death,[47] our findings show that breviscapine (50 μg/l) markedly attenuated elevation of intracellular calcium concentration induced by H_2O_2 (150 μM), suggesting that breviscapine can also relieved the cell toxicity induced by free radicals *via* preventing the intracellular calcium overloading.

It is well-established that some drugs can improve the learning and memory of AD or VD patients by modulating classic neurotransmitters. For example, tacrine produces its therapeutic effect mainly by inhibiting cholinesterase and increasing the concentration of acetylcholine in VD, as discussed above. Memantine, a low affinity antagonist to glutamate NMDA receptors, produces its therapeutic effect mainly by preventing excitatory neurotoxicity in VD.[48] On the other hand, numerous medications such as antioxidants, radical scavengers and calcium antagonists have been approved for the treatment of VD around the world.[49] In the line of this, our present study demonstrated that the effect of breviscapine on ischemic vascular dementia in rats could be due to its antioxidant action. However, the present results do not exclude the possibility that other mechanisms participate in the action of Breviscapine. Further studies need to be conducted.

In conclusion, the present study demonstrated that breviscapine improved chronic cerebral ischemic induced cognitive impairment, attenuated neuron injuries and inhibited intracellular calcium overloading. These findings provide experimental evidence for the application of breviscapine in the treatment of vascular dementia.

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