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ORIGINAL RESEARCH

Cerebroprotein Hydrolysate-I Inhibits Hippocampal Neuronal Apoptosis by Activating PI3K/Akt Signaling Pathway in Vascular Dementia Mice

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Introduction: Vascular dementia (VaD), one of the brain injuries, is difficult to be cured, so it is important to take active neuroprotective treatment after its occurrence. Many studies have shown that apoptosis serves an important role in VaD occurrence; therefore, inhibition of apoptosis may contribute to the recovery of neurological function after VaD occurrence. Cerebroprotein hydrolysate-I (CH-I), a neuropeptide preparation which consists of several amino acids and small molecular peptides as the main active constituent, is extracted using a method similar to cerebrolysin (CBL) which has neuroprotective and neurotrophic effects.
Methods: In the present study, a VaD model which was constructed using bilateral common carotid artery occlusion (BCCAO) in Kunming mice was applied to examine the neuroprotective effects of CH-I.
Results: The results show that CH-I treatment could attenuate the decrease of learning and memory ability, cell apoptosis in the hippocampal CA1 region and inhibit the activation of caspase-3 and caspase-9 in VaD mice. Furthermore, CH-I treatment could also upregulate Bcl-2 protein levels and activate PI3K and Akt.
Discussion: We speculate that CH-I may induce a neuroprotective effect activating PI3K/Akt signaling pathway in VaD mice.

Keywords: vascular dementia, cerebroprotein hydrolysate-I, apoptosis, PI3K/Akt

Introduction

Vascular dementia (VaD) is one of the brain injuries caused by cerebrovascular diseases, including ischemic injury and hemorrhagic injury,[1] and has been the second most common form of dementia.[2] By now, there is no exact definition of VaD,[3] but decreased thinking ability caused by decrease of cerebral blood[4] supply is referred to as the recognized symptom for identification of VaD.[5]

Because of the low cure rate, reducing occurrence and development of VaD that may be achieved by preventing the risk factors such as hypertension, smoking,[5] diabetes,[6] hypercholesterolemia,[7] atherosclerosis[8,9] and anxiety[10] is extremely important for VaD treatment. Numerous studies have proved that, after VaD occurrence, extensive cell apoptosis leads to neuronal deaths over time.[11–15] Therefore, inhibiting neuronal apoptosis may be an effective way to treat VaD.

Studies in recent years have shown that the brain protective agents can improve the symptoms of Alzheimer's disease and stroke, and have a certain effect on improving the cognitive ability and intelligence of patients.[16,17] Cerebroprotein

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hydrolysate-I (CH-I), a neuropeptide preparation which consists of lysine, leucine, proline, isoleucine, phenylalanine, proline, methionine, tryptophan and other amino acids and small molecular peptides as the main active constituent, is extracted using a method similar to Cerebrolysin (CBL) which has neuroprotective and neurotrophic effects.[18] In a recent study, it was found that CH-I mitigated stroke-induced white matter injury and facilitated axonal plasticity in the late stage after stroke.[19] CBL can improve the cognitive ability and general function of VaD patients,[20] attenuate apoptosis of thalamic neurons[21] and improve the neurological function in middle cerebral artery occlusion (MCAO) rats.[22] Therefore, we speculate that CH-I and CBL may have similar neuroprotective effects.

In the present study, the effect of CH-I in VaD mice was explored.

Materials and Methods

Animals

Male Kunming (KM) mice weighing 25–30g were obtained from Pengyue Experimental Animal Breeding Co., Ltd. (SCXK 20190003) and maintained under a 12 h light/dark cycle with free access to water and food. The National Institutes of Health Guide for the Care and Use of Laboratory Animals was used as guidance in designing all animal-related studies. Approvals for the study were acquired from the Ethics Committee of Qingdao University Medical College (QYFY WZLL 26274).

Reagents

CH-I was obtained from Hebei Zhitong Biopharmaceutical Co., Ltd. (Hebei China). Cerebrolysin (CBL) was purchased from EVER Neuro Pharma GmbH, Austria. TUNEL Apoptosis detection kits were purchased from Boster Biological Technology Co., Ltd. CA, USA. Rabbit anti-mouse Akt (8200S), p-Akt (8200S), PI3K (4292S), p-PI3K (4228S), and anti-rabbit IgG HRP-linked antibody (7074P2) were purchased from Cell Signaling Technology Co., Ltd. Bcl-2 (ab182858) was purchased from Abcam, Cambridge, UK. caspase-3 Caspase-3 (AF6311), caspase-9 (AF6348), ECL Western Blotting Substrate were purchased from Affinity Co., Ltd. USA. BCA protein concentration determination kits were purchased from Biosharp Life Science Co., Ltd. USA.

Behavioral Tests

The method described by Zhang et al[23] and Wei et al[24] served as the reference. In the present study, a Y-maze electric stimulator (Institute of Materia Medica Chinese Academy of Medical Science) was used to train and test mice's learning and memory abilities.

Mice's memory ability was tested 24 h after training using the following method: record the number of correct responses and take the correct rate as the testing score (correct number/10×100%). Higher accuracy rate means better memory ability for mice.

Surgery and Model Construction

The transient bilateral common carotid artery occlusion (BCCAO) surgery was performed as previously described and minor modifications were made on this basis.[25,26] Briefly, the mice were anesthetized with chloral hydrate (460 mg/kg i.p.). After skin disinfection, a midline anterior neck incision was made, the bilateral common carotid arteries were carefully separated from the adjacent vagus nerve and were locked by aneurysm clips for 20 min, followed by a release for 10 min, and this operation was repeated two times. The state of the mouse and suture were observed after the cycle was completed. Only the left and right common carotid arteries were separated and the aneurysm clips were not used in sham-operated animals.

Experimental Groups and Intervention

The 60 healthy adult KM mice were randomly divided into sham operation group (10 cases) and BCCAO operation group (50 cases). The mice under BCCAO operation were randomly divided into five groups: VaD model group (VaD), low dose CH-I administration group (CH-I-L, 10mg/kg), medium dose CH-I administration group (CH-I-M, 20mg/kg), high dose CH-I administration group (CH-I-H, 30mg/kg) and CBL administration group (CBL, 10mg/kg), and each group included 10 mice. After 7 days, the learning and memory abilities of all mice were tested by the Y-maze. The mice in the sham group and the VaD group were injected with 0.5 mL of 0.9% normal saline every day, the mice in CH-I-L, CH-I-M and CH-I-H groups were administered CH-I at 10, 20, 30 mg/kg, respectively, and the CBL group was administered CBL at 10 mg/kg. All mice were treated with intraperitoneal injection, 0.5 mL/d, for 4 consecutive weeks. After the last administration, the surviving mice (n=8) of each group were subjected to behavioral tests.

HE Staining

Four mice in each group were anesthetized by intraperitoneal injection of 10% chloral hydrate, and the heart was perfused with 20 mL of normal saline and 20 mL of 4% paraformaldehyde. The brain was completely removed, dehydrated, waxed, embedded, and serial coronal sections (8 μm) patch spared. The paraffin sections were taken, deparaffinized and hydrated, stained with hematoxylin (G1120, Solarbio, CN) for 3 min, differentiated for 10 s, soaked in tap water for 15 min, stained with eosin (G1120, Solarbio, CN) for 1 min, and the color development was stopped in tap water. Conventional dehydrated, transparent, and neutral balsam seals were applied.The hippocampus structure was observed under a light microscope, and the nucleus and the cytoplasm showed blue and red, respectively. Five non-overlapping fields on each slice were recorded by optical microscope (IX70, OLYMPUS, JPN). The degree of damage is expressed by the denatured cell index (DCI, number of denatured cells/total number of cells).

Nissl Staining

The paraffin sections were taken, deparaffinized and hydrated, then the sections were put into cresyl violet stain (G1430, Solarbio, CN) at 56°C for 1 h. After being soaked in deionized water, the sections were put into Nissl Differentiation (G1430, Solarbio, CN) at room temperature for 1 min. Conventional dehydrated, transparent, and neutral balsam seals were applied. The hippocampus structure is observed under a light microscope. The positive neurons were clear and intact, and intracellular dyeing is blue-violet. The degree of damage is expressed by the denatured cell index (DCI, number of denatured cells/total number of cells).

TUNEL Staining

TUNEL assay kits were used for the analysis of cell apoptosis according to the manufacturer's instructions. The cells with brown particles in the nucleus under light microscope are considered as apoptotic cells. Part of the sections added 0.1M PBS as probe, and there was no positive reaction. Four serial sections from each mouse were taken and brown cell numbers in four random regions were counted in each section.

Western Blotting

Four mice were taken from each group and 10% chloral hydrate was injected intraperitoneally for anesthesia, and the brains were taken after infusion of 20 mL normal saline through the heart. The hippocampus tissue was taken on ice and lysed with RIPA lysis buffer and then centrifuged at 4°C for 15 min. For each group, 20 μg protein determined using BCA assay was separated by SDS–PAGE gel (10–15%) and transferred to a PVDF membrane. After blocking with 5% non-fat milk at room temperature for 1 h, the membranes were incubated with primary antibodies at 4 °C overnight. The signals were detected using an enhanced chemiluminescence system. The gel imaging analysis system measures the gray value of each band, and expresses the protein level in terms of relative value of protein (RVP, gray value of target protein/gray value of internal reference protein).

Statistical Analysis

Statistical analysis was performed with SPSS 20.0 software, and the data were presented as mean ± standard deviation ($\bar{X}$ ± S), using Student's *t*-test for two-group comparisons or one-way ANOVA for multiple-group comparisons. A p-value of less than 0.05 was considered significant.

Results

CH-I Improved Learning and Memory Ability of VaD Mice

As shown in Table 1, there was no significant difference in the number of learning times required to reach the target in each group ($p>0.05$). After modeling, the number of attempts significantly increased in all modeling mice compared with sham group ($p<0.01$), indicating that the learning ability of mice significantly decreased, that is, the modeling was successful. Compared with sham group, the number of attempts in VaD group, CH-I-H group

Table 1 Comparison of Learning Ability of Mice in Each Group ($\bar{X}$±S)

	Before Modeling	After Modeling	After Treatment
Sham	29±4.22	20.88±7.57	27.5±4.84
VaD	29.1±7.92	40.1±7.4**	47.25±5.18**
CH-I-L	29±6.32	39.75±6.41**	34.88±5.08##
CH-I-M	30±8.76	42.25±8.35**	34.88±5.08##
CH-I-H	27.9±8.33	43.38±4.72**	38.5±7.07*#
CBL	27±8.43	41±5.35**	37.25±7.44*##

Notes: *$p<0.01$ vs sham; **$p<0.01$ vs sham; #$p<0.05$ vs VaD; ##$p<0.01$ vs VaD.

(P<0.05) and CBL group (p<0.01) significantly increased. Compared with the VaD group, the number of attempts of CH-I-treated and CBL group mice significantly decreased (p<0.05 and p<0.01, respectively).

As shown in Table 2, compared with sham group, the success rate of each model mouse significantly decreased (p<0.05). Compared with the VaD group, the success rate of CH-I-L and CH-I-M groups significantly increased (p<0.01), but there was no significant difference between CH-I-H or CBL group and VaD group.

HE Staining Shows That CH-I Reduced the Number of Abnormal Nerve Cells in the Hippocampal CA1 Regions of VaD Mice

As shown in Figure 1, compared with sham group, the denaturation of hippocampal neurons in VaD group significantly increased (p<0.01). The hippocampal CA1 region of mice in the VaD group displayed increased abnormal cell morphology, with reduced volume, darker staining, loose structure, disordered arrangement, and nuclei solid shrinkage. After treatment, the number of degenerated hippocampal cells in CH-I-treated group and CBL group significantly decreased compared with the VaD group (p<0.01). The therapeutic effect of CH-I-L group and CH-I-M group was better than that of CH-I-H group (p<0.05).

CH-I Reduces the Number of Apoptotic Cells in the Hippocampal CA1 Region of VaD Mice

As shown in Figure 2, compared with sham group, the number of positive hippocampal apoptotic cells in VaD group increased significantly (p<0.01). After treatment, the number of apoptotic cells in the hippocampal region of mice in each treatment group decreased, and the difference was statistically significant compared with VaD group (p<0.01). Compared with CH-I-H group, CH-I-L group had better treatment effect (p<0.05). However, compared with CBL group, the treatment effect of CH-I-L group and CH-I-M group showed no statistical difference (p<0.05).

Table 2 Comparison of Memory Ability of Mice in Each Group (%) ($\bar{X}$±S)

	Before Modeling	After Modeling	After Treatment
Sham	0.9±0.09	0.89±0.08	0.86±0.09
VaD	0.88±0.1	0.5±0.16**	0.39±0.15**
CH-I-L	0.9±0.08	0.46±0.14**	0.69±0.15##
CH-I-M	0.87±0.08	0.48±0.14**	0.7±0.13##
CH-I-H	0.85±0.08	0.45±0.09**	0.53±0.16**
CBL	0.88±0.08	0.53±0.14**	0.55±0.15**

Notes: **p<0.01 vs sham; ##p<0.01 vs VaD.

Effect of CH-I on PI3K, p-PI3K, Akt and p-Akt in the Hippocampus of VaD Mice

As shown in Figure 3, compared with sham group, the ratios of p-PI3K/PI3K and p-Akt/Akt significantly decreased in VaD group (p<0.05). After treatment, the ratios of p-PI3K/PI3K and p-Akt/Akt significantly increased in CH-I-treated and CBL groups compared with VaD group (p<0.01). Moreover, compared with CBL group, the treatment effect of CH-I-L group and CH-I-M group showed no statistical difference (p<0.05).

Effect of CH-I on Caspase-9, Caspase-3, Bcl-2 and Bax in Hippocampus of VaD Mice

As shown in Figure 4, compared with sham group, the protein levels of caspase-9 and caspase-3 in hippocampus of VaD mice significantly increased (p<0.01), and Bcl-2 protein levels significantly decreased (p<0.01). After treatment, the protein levels of caspase-9 and caspase-3 in CH-I-treated group were significantly reduced compared with VaD group (p<0.01), but there was no significant change in the protein levels of CBL group (p>0.05). Compared with VaD group, the protein levels of Bcl-2 in CH-I-L group and CH-I-M group significantly increased (p<0.01).

Discussion

Vascular dementia is one of the sequelae of severe ischemic stroke. VaD development is often associated with chronic cerebral ischemia.[27] In experimental studies, vascular occlusion is usually used to simulate the process of chronic total cerebral ischemia in patients. In the present study, the modified method of bilateral common carotid artery occlusion was adopted for animal modeling. During the modeling period, the mice showed symptoms such as cyanosis of mouth and extremities, and rapid heart rate, etc. The behavioral test after modeling showed that the learning and memory ability of the model mice decreased. However, after four weeks of continuous CH-I administration, the learning and memory ability of the

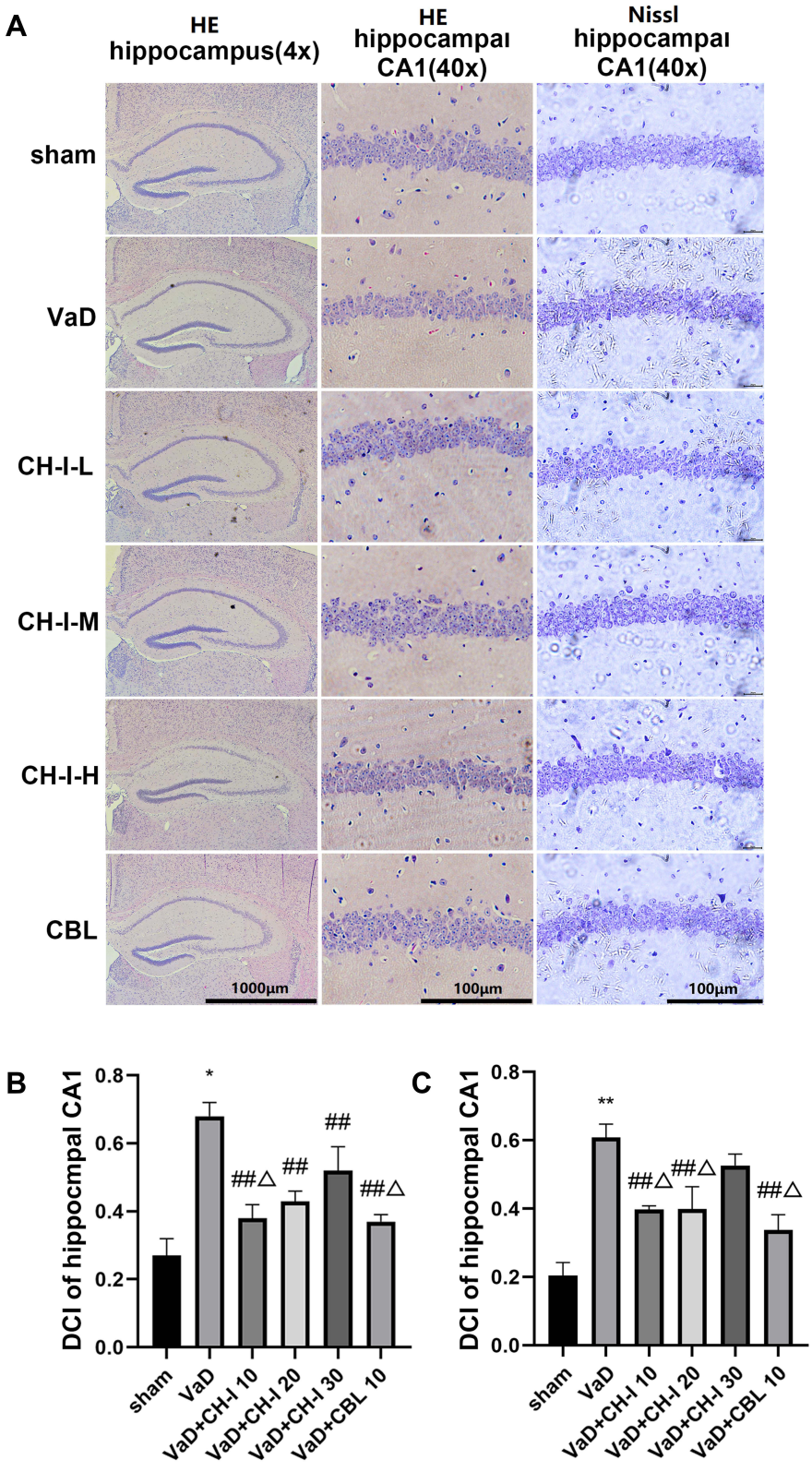

Figure 1 Observation results of different multiples of HE and Nissl staining in the hippocampus of mice in each group ($\bar{X}$±S, n=4). (**A**) The results of HE staining (4×, 40×) and Nissl staining (40×) in the hippocampus of each group; (**B** and **C**) Comparison of degeneration cell index of hippocampus CA1 of mice in each group; *p<0.05 vs sham; **p<0.01 vs sham; $^{\#\#}p$<0.01 vs VaD; $^{\triangle}p$<0.05 vs CH-I-H.

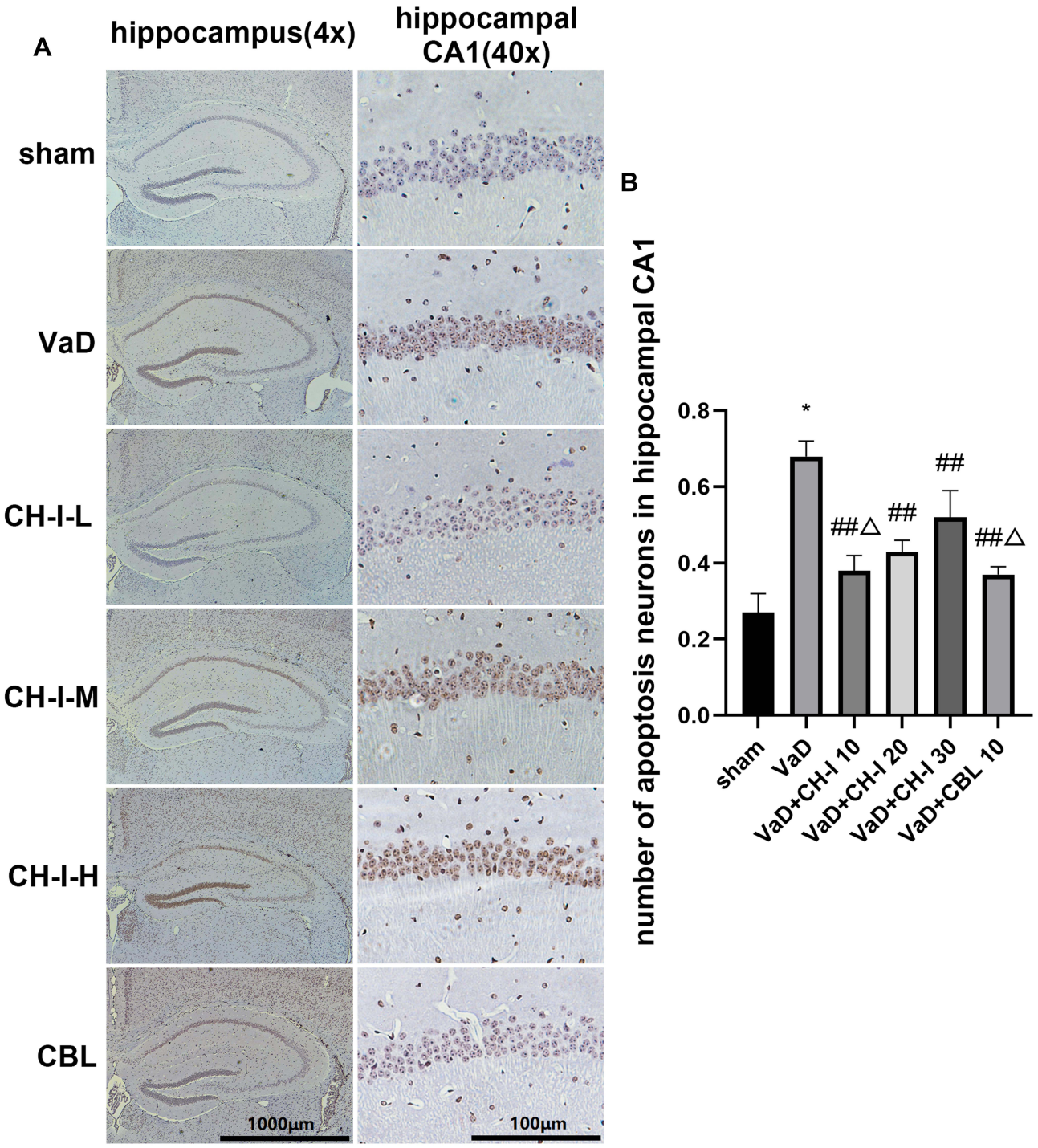

Figure 2 Comparison of the number of neuronal apoptosis in the hippocampus of each group of mice ($\bar{X}$±S, n=4). (**A**) TUNEL staining (4×, 40×) results of the hippocampus of each group of mice; (**B**) Comparison of the number of apoptotic neurons in the hippocampus CA1 of each group. *p<0.05 vs sham; $^{\#\#}p$<0.01 vs VaD; $^{\triangle}p$<0.05 vs CH-I-H.

mice in each administration group was better than that of the model group. The anterior hippocampus which is responsible for plot memory and visual scene perception in brain tissue is very sensitive to ischemia.[28,29] Studies have shown that, after clipping and reperfusion of bilateral common carotid artery, significant loss of neurons was found in the hippocampus CA1 region.[30] In the present study, the results show that a significant decrease in number in the nerve cells in hippocampal CA1 regions of VaD mice was accompanied by morphological modifications, such as abnormal morphology, nucleolar shrinkage and disordered arrangement. After CH-I treatment, the number

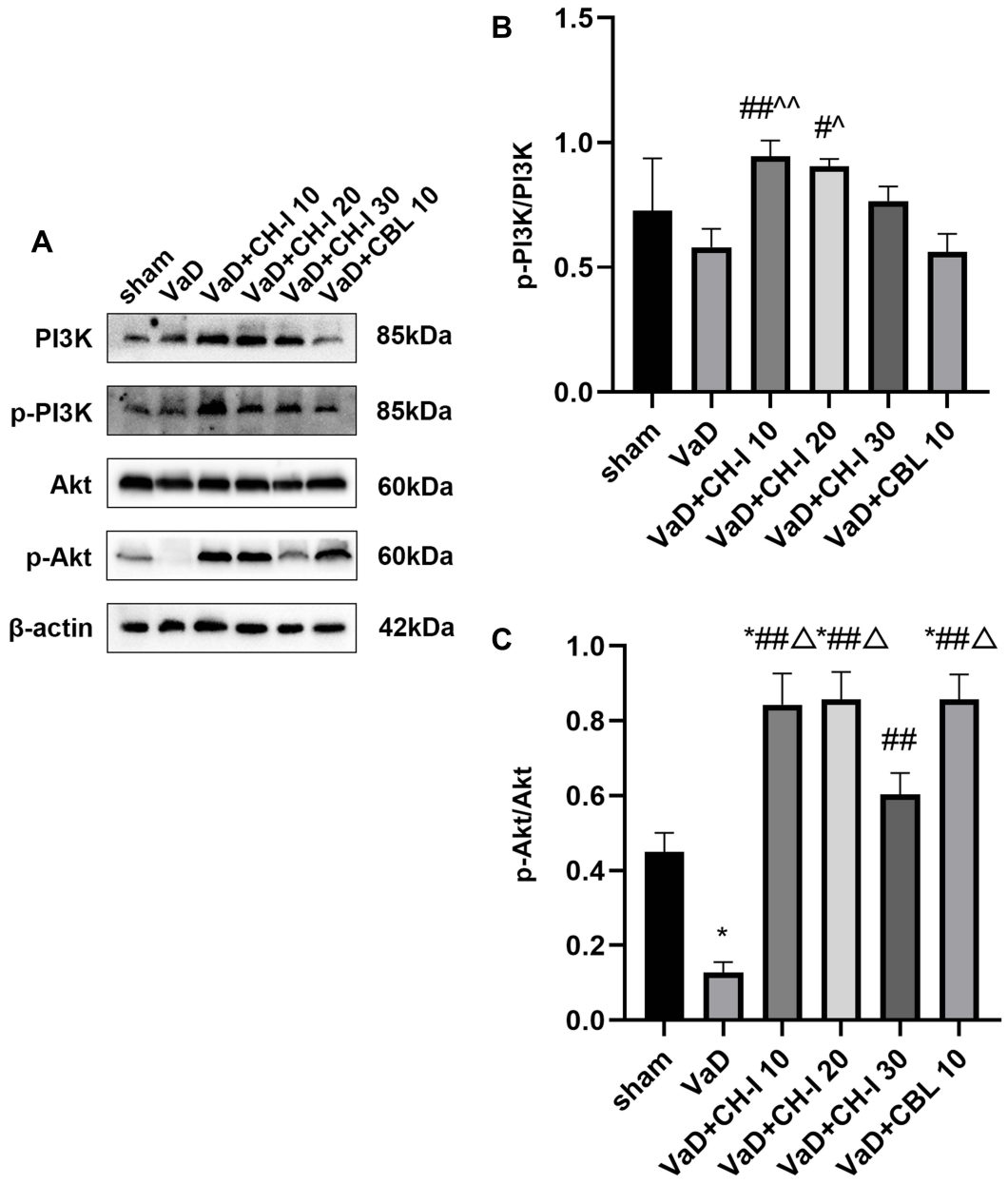

Figure 3 The expression of Akt, p-Akt, PI3K, and p-PI3K in the hippocampus of each group of mice ($\bar{X}$±S, n=4). (**A**) The expression of Akt, p-Akt, PI3K, and p-PI3K in the hippocampus of each group; (**B**) The relative protein expression of p-Akt/Akt in the hippocampus of each group; (**C**) The relative protein expression of p-PI3K/PI3K in the hippocampus of each group. *p<0.05 vs sham, $^{\#}p$<0.05 vs VaD, $^{\#\#}p$<0.05 vs VaD, $^{\triangle}p$<0.05 vs CH-I-H, $^{\wedge}p$<0.05 vs CBL, $^{\wedge\wedge}p$<0.01 vs CBL.

of abnormal cells decreased, and the nerve cells in hippocampal CA1 regions were protected.

Apoptosis, a type of programmed cell death, is used to accurately regulate the number of cells and eliminate unwanted and potentially dangerous cells.[31] In the present study, TUNEL assay was used to label the apoptotic cells in the hippocampal region, and it was found that the number of positive cells in the hippocampal CA1 regions increased in VaD mice, and the number of positive cells in the two regions decreased significantly after CH-I treatment, indicating that CH-I can inhibit the apoptosis in hippocampal nerve cells. Intrinsic apoptotic pathways are activated by various intracellular stimuli and depend on apoptotic bodies. Aspartic protease 9 (caspase-9) is one of the components of these apoptotic bodies. In mammalian cells, caspases are the main executor of apoptosis, and their activation is strictly controlled by the B-cell lymphoma-2 (Bcl-2) family protein. A series of members of the family of the Bcl-2 by adjusting the mitochondrial membrane permeability of the release of cytochrome-c

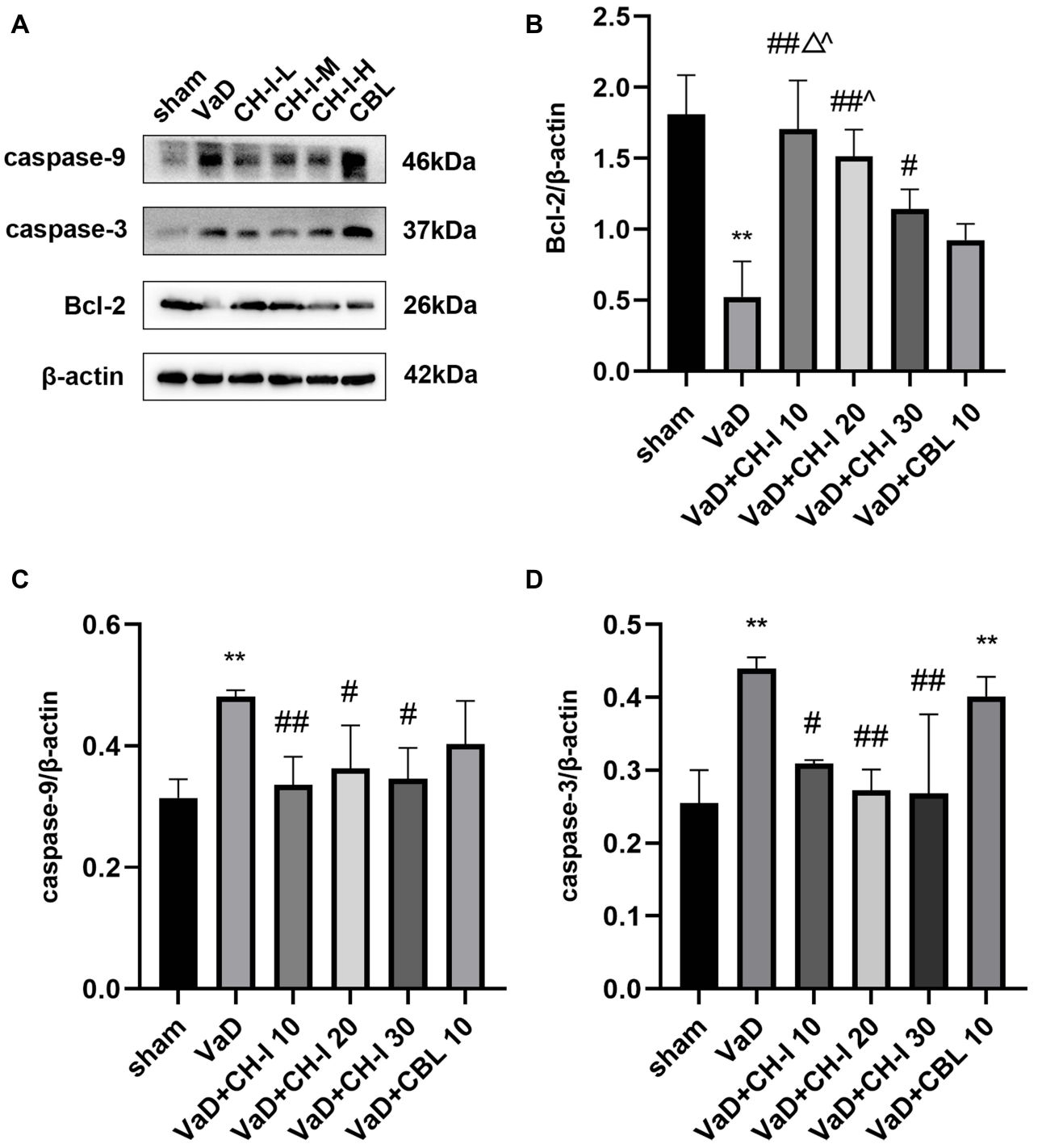

Figure 4 The expression of caspase-9, caspase-3, and Bcl-2 in the hippocampus of each group of mice ($\bar{X}$±S, n=4). (**A**) The expression of caspase-3, caspase-9, and Bcl-2 in the hippocampus of each group; (**B**) The relative expression of caspase-9 in the hippocampus of each group; (**C**) The relative expression of caspase-3 in the hippocampus of each group; (**D**) The relative expression of Bcl-2 in the hippocampus of each group. $^{**}p<0.01$ vs sham, $^{\#}p<0.05$ vs VaD, $^{\#\#}p<0.05$ vs VaD, $^{\triangle}p<0.05$ vs CH-I-H, $^{\wedge}p<0.05$ vs CBL.

(Cyt-c), and apoptosis protease activating factor-1 (Apaf-1) interaction, cause the activation of caspases-9, leading to subsequent characteristics of cell apoptosis, cell nucleus fragmentation, chromatin condensation, and chromosomal DNA fragmentation.[32] In the present study, apoptosis of neurons was confirmed by the results of the TUNEL assay and the protein levels of Bcl-2, caspase-9 and caspase-3 in the hippocampal tissues. A phosphoinositide 3 kinase (phosphoinositide 3 kinase, PI3K)/protein kinase B (protein kinase, PKB/Akt) signaling pathway, which is

important to promote nerve growth and survival pathways, has been confirmed in many aspects associated with inhibiting apoptosis[33,34] and promoting neuronal survival.[35] The results of this study showed that the p-Akt/Akt ratio in the hippocampal tissue of VaD mice was significantly reduced, while the P-Akt/Akt ratio in each group treated by CH-I was significantly increased, indicating that CH-I can activate Akt to produce P-Akt and enable it to play an important role in promoting the survival of nerve cells.

In conclusion, our results show that CH-I can up-regulate Bcl-2 protein and down-regulate the expression of caspase-9 and caspase-3 protein in the hippocampal tissues of VaD mice by activating Akt, and inhibit the apoptosis of hippocampal nerve cells, thus significantly improving the learning and memory functions of VaD mice. The therapeutic effect was within the dose range of this experiment, and there was no obvious dose–response relationship. The effect of low dose was the best. Compared with the low dose of CH-I, the same dose of CBL showed the same intensity of up-regulation of p-Akt/Akt and inhibition of apoptosis of hippocampal nerve cells. However, CBL did not improve the expression of caspase-9 and caspase-3 protein in VaD mice, and even further down-regulated the level of Bcl-2 protein. This may be related to the failure of CBL to improve memory impairment in VaD mice. Although the total amount of small peptides in CH-I and CBL is the same, the types and proportions of small peptides in each are still unknown. Further chemical and pharmacodynamic studies of the components of CH-I peptides will help to develop more effective drug candidates for the treatment of VaD.

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Disclosure

The authors declare no conflicts of interest for this work.

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