Etidronate rescues cognitive deficits through improving synaptic transmission and suppressing apoptosis in 2-vessel occlusion model rats

Running title: Neuroprotective effects of Etidronate

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Abbreviations used: Ca²⁺, calcium; CaMKII, calcium/calmodulin -dependent protein kinase II; ET, Etidronate; LTP, long-term potentiation; MDA, lipid peroxidation malondialdehyde; MWM, Morris water maze; PBS, phosphate buffer saline; SOD, superoxide dismutase; TBS, theta burst stimulation; TBST, tris-buffered saline and Tween 20; VD, vascular dementia; 2-VO, permanent bilateral common carotid artery occlusion.
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Abstract: Vascular dementia (VD) is a neurodegenerative disorder caused by the reduction of cerebral blood flow, which shows a progressive cognitive impairment. In our previous study, we found that Etidronate (ET) showed neuroprotective effects against glutamate-injured PC12 cells and thus, in this study, we aimed to observe the effects of ET regarding the learning and memory impairment and their related mechanism in 2-vessel occlusion (2VO) model rats. These rats were administrated by permanent bilateral common carotid artery occlusion to induce the VD model. Two weeks later, 2VO model rats were treated with ET (10ml/kg/day i.p.) for one week. Results showed that ET improved the spatial learning and memory function in 2VO rats conducted by a Morris water maze experiment. Also, a reduced long-term potentiation (LTP) was rescued by ET treatment in 2VO rats. Moreover, the LTP-related proteins calcium/calmodulin-dependent protein kinase II (CaMKII), NMDAR 2B and PSD95 were all up-regulated after the ET treatment. The tests also showed that after the ET treatment the level of oxidative stress was lowered and so as to the the levels of malondialdehyde and superoxide dismutase in 2VO rats. Furthermore, ET displayed a better anti-apoptosis ability through detecting the levels of Bcl-2 and Bax protein and TUNEL-positive cells. In conclusion, ET showed neuroprotective effects on 2VO rats through rescuing spatial working memory deficits. And a possible mechanism maybe suggested in between the increased synaptic transmission, the reduction of oxidative stress, and inhibition apoptosis.

Introduction

Vascular dementia (VD) is considered as a progressive cognitive impairment and caused by the reduction of cerebral blood flow (Grantham & Geerts 2002). It is a progressive aging-related disease (Giacobini 2004). And along with the aging of the population increase. More and more old people suffer from VD. Therefore VD needs
an urgent attention and treatment. At present, there are some drugs reported for therapeutic treatment for VD (Xu et al. 2012, Dai et al. 2007, Liu et al. 2016, Luo et al. 2016). However, none of these specific drugs effective and VD is still remained none-curable. Thus, the searching for new effective drugs is needed to prevent the progressive cognitive and memory impairment caused by VD.

In human brain, learning and memory can be affected by many signal pathways. Calcium (Ca\textsuperscript{2+}) acts as a cellular second messenger and plays an extremely important role to promote synaptic transmission (Sato et al. 2003). CaMKII is a major calcium activated protein, and which interacts with a large number of synaptic proteins, such as glutamate receptor, the NMDAR 2B. Phosphorylated CaMKII promotes synaptic transmission and in turn, increases the ability of learning and memory (Wang et al. 2013, Lisman et al. 2002). Some research reported that the level of phosphorylated CaMKII is lower in VD rats compared to that of normal (Ge et al. 2015a), and therefore, there is a need to seek the drug to enhance the level of phosphorylated CaMKII.

Disodium Etidronate (ET) is the first generation of bisphosphonates as therapeutic drug to treat osteoporosis and bone diseases providing excellent intercellular Ca\textsuperscript{2+} binding affinity. Our previous research paper proved for the first time, that ET regulated or increased the concentration of Ca\textsuperscript{2+} both inside and outside of PC12 cells, which significantly reduced the reactive oxygen species generation and cell apoptosis in glutamate-injured PC12 cells (Li et al. 2016). Prior to present study, we had hypothesized that ET would provide excellent improvement on learning and memory impairment in 2-vessel occlusion (2VO) model rats. The molecular mechanisms may be involved in the activation of Ca\textsuperscript{2+} channels associated with the increased expression of NMDAR 2B and PSD95. Meanwhile, ET can suppress the excessive oxidative stress so as to reduce the incidence of neuronal apoptosis in hippocampus. Our aim is to reveal if ET can be considered as a valid potential neuroprotective drug to treat vascular dementia.
Materials and methods

Materials and reagents

ET (purity>98%) was purchased from TOKYO CHEMICAL INDUSTRY CO, LTD. (TOKYO, JAPAN). Anti-β-actin, anti-NMDAR2B antibody, anti-PSD95 antibody, anti-CaMK II (phospho) antibody, anti-CaMK II antibody, anti-Bax antibody and Bcl-2 antibody were acquired from Abcam (Cambridge, UK). Superoxide Dismutase (SOD) assay kit, lipid peroxidation malondialdehyde (MDA) assay kit, and TUNEL apoptosis assay kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Animals

Adult male Wistar rats (200-220g) were acquired from the Experiment Animal Centre of Chinese Academy of Medical Science. The rats were raised in Animal House of Medical School of Nankai University and were housed in cages of the appropriate size with access to food and water. Prior to surgery, they had 3 days to acclimatise to a new environment, which was controlled at 22±2℃ temperature, 50-60% humidity and under a 12-h artificial light-dark cycle. Any endeavours were made to keep a minimum of animal number and suffering. All animal experiment procedures were authorised by the Committee for Animal Care at Nankai University and coincided with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

2-vessel occlusion (2VO) model and ET treatment

Rats were divided into three groups randomly (n=6 rats/group): sham group, 2VO group and 2VO+ET group. Rats were in ambrosia but had free access to water for 12 hours before surgery and were performed operations under sterile condition. They were anesthetized by intraperitoneal injection (i.p.) of 10% chloral hydrate (4ml/kg) and then were fixed on the rat boards. The VD model was conducted by permanent bilateral common carotid artery occlusion and the procedure of 2-VO referring to a
previous description but with small amount of modifications (Yang et al. 2014). The sham group received the surgery without handling the bilateral common carotid arteries. After the operation, every rat was put on an electric blanket in a sawdust cage to maintain its body core temperature between 36℃ and 37℃ until regaining consciousness, and then they were reared in the Animal House, with food and water available to them.

The 2VO+ET group: Two weeks after 2-VO surgery, rats were treated with ET (20mg/kg/day i.p.) dissolved in saline (Kawabata et al. 2006). In sham and 2VO group, rats were injected the same dosage of saline intraperitoneally once a day for 7 consecutive days.

**Morris water maze test**

Seven days of treatment, each group of rats were trained and tested in Morris water maze (MWM, RB-100A type, Beijing, China) in order to evaluate spatial learning and memory. This equipment consisted of a circular vat (150cm in diameter, 50cm in height), a circular platform (10cm in diameter, 30cm in height) and a set of a photographic device for recording the swimming trajectory of the rats. The vat, divided into four quadrants (Ⅰ-Ⅳ), was filled with water, which was maintained at 25℃ and dyed by a non-toxic black ink to conceal the platform. The platform was placed in the middle of quadrant, 2cm below the water surface.

In this study, the test consisted of two connected phases, the place navigation phase and the spatial probe phase. During place navigation phase, rats were trained twice per day (eight-hour interval and four trials once) for 5 consecutive days. In each round, rats were faced the pool wall and were put into the water gently from the midpoint of the wall edge in a random order. Then swimming trajectories, time took to find the platform (escape latency) and swimming speeds were monitored and recorded. Recording would stop automatically 2s after rats found the platform. If a rat failed to find the platform within 60s, we guided it to rest on the platform for 10s, and its escape latency was recorded as the 60s. The interval between each round was more than 10min. The spatial probe test was performed once at least 24h after the last trial
of the navigation training. In this phase, the platform was removed. Rats were put into water from the midpoint of the wall edge in quadrant IV and swam the 60s freely. The percentage of time spent in the target quadrant was recorded.

**In vivo electrophysiological test**

Following the MWM test, the long-term potentiation was measured by *in vivo* electrophysiological test. First, the rat was narcotized with 30% urethane (0.45ml/kg, i.p.), and then positioned on a stereotaxic frame (SR-6N; Narishige, Japan) for surgery, and observation according to former protocols (Gao et al. 2015). To insert a stimulating electrode and a recording electrode, a proper incision on the scalp was cut to expose the skull and drill a hole. In stereotaxic coordinates, the bipolar stimulating electrode was positioned in hippocampal Schaffer collaterals (4.2mm posterior to bregma, 3.5mm left to midline, 2.3-2.6mm below the dura). The recording electrode was implanted in the stratum radiatum of the hippocampal CA1 area (3.5mm posterior to bregma, 2.5mm left to midline, 2.0-2.2mm below the dura). The test stimuli were delivered to Schaffer collaterals every 30s at an intensity, which could evoke a response of 70% of its maximum, within a range of 0.3-0.5mA. Sampling was made under single-pulse stimulation (0.05Hz) for 30 min as the baseline. And then, a theta burst stimulation (TBS, 30 series of 12 pulses at 200Hz) was transmitted to induce LTP. The single-pulse recording was recovered every 60s for 60min following TBS.

**Hematoxylin/Eosin (HE) staining**

A portion of rats was subjected to perfusion fixation of rat brain tissue through *in vivo* heart. Phosphate buffer saline (PBS) was infused until the observation of the clearing of the running fluid, followed by 4% paraformaldehyde (PFA) perfusion. Once brains were removed, they were immersed in PFA at 4℃ over 24 hours. Whereafter, brains were dehydrated through a gradient sucrose solution and were embedded in OCT compound (Tissue-Tek, Miles) for frozen section (Leica CM 1850, Leica Instruments), and sectioned at 10μm thickness. For pathological changes in hippocampal CA1 and CA3 region, the sections were stained with Celestine blue and acid fuchsin.
Microscopic images were taken, in which rectangular area of equal size (0.6mm*0.5mm) were randomly chosen from three different sections of one sample, and the average was taken. Survival pyramidal cells in hippocampal CA1 and CA3 region were counted respectively.

**Measurement of MDA levels and SOD activity**

After the electrophysiological test, hippocampus was quickly removed from brains and stored at -80°C immediately. As necessary, total proteins in the hippocampus were extracted by tissue homogenate method, and protein concentrations were determined by BCA Protein Assay Kit (Beyotime, Shanghai, China). After that, MDA levels and SOD activity of hippocampal tissues were measured with commercial kits according to the manufacturer’s instructions.

**Tunnel staining and immunofluorescence staining**

TUNEL procedures were applied to frozen brain sections using the TUNEL Apoptosis Assay Kit, according to the manufacturer’s protocol. Apoptotic cells were detected as localised bright green cells in a green background under a laser scanning confocal microscope (LSCM, Olympus FV1000, Japan).

For the immunofluorescence staining, the frozen brain sections were washed three times with PBS, then were incubated with 0.5% Triton for 10min at room temperature, followed by washing with PBS for three times, then blocked with 10% goat serum for 1 hour at room temperature. And followed by incubation with primary antibodies (anti-PSD95: 1:1000) at 4°C overnight. After primary antibody incubation, sections were washed three times with PBS, followed by incubation with the 594-conjugated anti-rabbit IgG secondary antibody (1:1000) for 1 h at room temperature. After secondary antibody incubation, sections were washed three times with PBS, followed by incubation with DAPI for 5 minutes at room temperature. Then sections were washed three times in PBS. Finally, sections were examined under a laser scanning confocal microscope (LSCM, Olympus FV1000, Japan). The
fluorescent intensity was quantified for randomly three fields of one sample by Image J software, and the average was taken.

**Western blot analysis**

Proteins were electrophoresed on a 8% or 13% SDS-PAGE gel (spacer gel, 80V, 30min; separation gel, 120V, 90min) and then electro-transferred to PVDF membranes. The PVDF membranes were incubated in the 5% skim milk for one hour at normal temperature to avoid non-specific binding and then were incubated with primary antibodies, which was diluted by 5% skim milk at 4°C overnight. After washed with Tris-buffered saline and Tween 20 (TBST), the PVDF membranes were incubated with secondary antibodies for 40min accurately. Subsequently, the PVDF membranes were washed with TBST again and immersed in equal parts developing fixing reagent A and B for one minute. Protein blots were exposed with a light exposure apparatus. Equivalent protein loading was ascertained by comparison of β-actin expression quantity on the same Western blot assay. The optical density ratio of every investigated protein and β-actin was analysed.

**Statistical analysis**

To estimate the sample size in each group, the standard deviation of the LTP level in rats subjected to permanent bilateral common carotid artery occlusion surgery was 6.5% in the previous studies. For our power calculation, we assumed an equal standard deviation in each group. We wanted to show a difference of 15% in the LTP level among the groups. With α=0.05, one-sided and a power of 99%, we needed minimum 6 rats per group. So, we employed 18 rats in this study. For all animal experiments, rat in each group was selected by completely randomized design. And double blinding method was used for group assignment and outcome assessment. The sample size (n) of each experimental group was described in each corresponding figure legend, and all experiments were repeated at least with three times. All results
were expressed as mean ± Standard Error Mean (S.E.M.). All data analysis was executed by SPSS 22.0, and cartograms were drawn by OrignPro 8.5. The statistical differences between 3 groups were analyzed by One-Way ANOVA and further Dunnet’s multiple comparisons and were considered significant if P<0.05.

Results

ET ameliorated spatial cognition in water maze task

For assessing the learning and memory function of ET, the five consecutive days of training trial were performed by MWM devices (Xu et al. 2010, Hosseinzadeh et al. 2012). In place navigation phase, the escape latency was significantly higher in the 2VO group compared with that of sham group (P < 0.05). These results indicated that spatial cognitive deficit existed in the 2VO group. And the treatment with ET decreased the escape latency, which demonstrated a partially recovery of the learning and memory capacity in the 2VO+ET group (P < 0.05, Fig. 1A). In spatial probe phase, the percentage of time in target quadrant was tested to evaluate the rate memory. Results revealed that rats suffered from 2VO procedure showed worse memory capacity with shorter mean spending time in the target quadrant (P < 0.05). In 2VO group, treatment with ET, the memory ability was significantly improved by spending more time in the target quadrant (P < 0.05, Fig. 1C and 1D). We also observed the swimming speed of the three groups, and showed no significant differences. These results eliminated the possibility that the learning and memory capacity differences were caused by movement disorders (Fig. 1B).

Effects of ET on the synaptic transmission of Schaffer collateral-CA1

In in-vivo electrophysiological test, recording LTP from Schaffer collaterals to CA1, was used to measure the synaptic plasticity and analyze learning and memory ability. The fEPSP slopes were obviously enhanced during one hour after a theta burst stimulation (TBS) than the fEPSPs baseline during the 30min before TBS (Fig. 2A). What’s more, the fEPSP slopes were remarkable lower in 2VO group than those in
sham group. The fEPSP slopes were evident improved in 2VO+ET group compared with those in 2VO group (Fig. 2B). As a result, the synaptic plasticity of rats suffering global cerebral ischemia got injury, and ET could reduce this damage.

**Effects of ET on histology of the CA1 and CA3 region**
Neurodegenerative pathologic findings, such as necrosis and loss of hippocampal neurones, generally appeared in rats with cerebral ischemia, so HE staining was chosen to investigate lesions of CA1 and CA3 pyramidal cells in the hippocampus. Pyramidal cells in CA1 and CA3 region were distributed orderly and arranged densely in the sham group. But those cells on the section from 2VO group had severe irregular and loose arrangement, with obvious karyoclasis, karyopyknosis and neuron loss. Compared with 2VO group, there were only slight morphology changes of pyramidal cells, and survival pyramidal cells in hippocampal CA1 and CA3 regions were significantly increased in 2VO+ET group (Fig. 3), which demonstrated that ET can reduce neuron loss.

**ET reduced oxidative stress level in hippocampus**
Global cerebral ischemia was certified to give rise to drastic oxidative stress, which manifested as incremental lipid peroxidation and descending antioxidant enzyme activities (Safwen *et al.* 2015). The level of enzymatic antioxidant activity and lipid oxidation were determined by SOD and MDA kit, respectively. Results indicated that SOD activity was dramatically dropped in the 2VO group compare to that of the sham group. And in accordant with the previous results, the administration of ET can markedly increase the SOD activity in the hippocampus (P <0.01, Fig. 4A). The result of MDA is opposite to SOD, as shown in Fig. 4B, MDA level was much higher in 2VO group compared with that of the sham group. The MDA concentration was decreased after treatment with ET in 2VO+ET group (P < 0.01).

**ET treatments prevented apoptosis and enhanced synaptic density**
A terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay was
performed to estimate neuronal apoptosis in the hippocampus. We found that the number of TUNEL-positive cells was significantly increased in the 2VO group than that of in the sham group (P<0.01). And a substantial decrease in the 2VO+ET group, suggesting that ET prevented a loss of neurones (P<0.05, Fig. 5A).

We performed postsynaptic density protein 95 (PSD95) immune-labelling to examine the synaptic functional recovery through ET treatment. The results showed that the lower intensity of PSD-95 in the 2VO group compared to the sham group (P<0.01). And a significant increase was found in the 2VO+ET group, suggesting that ET rescued the synaptic damage (P<0.05, Fig. 5B).

**Western blotting analysis**

ET up-regulated the phosphorylation rates of CaMKII, NMDAR 2B and PSD95 in 2VO rats. As shown in Fig. 6A and Fig. 6B decreased ratios of p-CaMKII/CaMKII, NMDAR 2B and PSD95 were observed in the hippocampus of the 2VO rats compared to those in the sham group (P < 0.01). Administration of ET significantly up-regulated the phosphorylation rates of CaMKII, NMDAR 2B and PSD95 in 2VO rats (P < 0.01 and P < 0.05 vs the 2VO group, respectively).

We also analyzed neuronal apoptosis, which was considered as an important pathological mechanism in the development of 2VO. As shown in Fig. 6C, a decrease was found in Bcl-2/Bax ratio compared with sham group (P < 0.01). And the treatment with ET significantly increased the Bcl-2/Bax ratio in 2VO rats (P < 0.01 vs. the 2VO group).

**Discussion**

VD is a neurodegenerative disorder that results in a progressive deficit in memory and cognitive function (Rammes 2009). The pathogenesis of this disease is related to the reduced synaptic transmission (Xing *et al.* 2016), the generation of reactive oxygen species (Luca *et al.* 2015) and neuronal apoptosis (Xu *et al.* 2016). In our previous study, we have proved that ET has a good neuroprotective effect on glutamate-injured
PC12 cells (Li et al. 2016). Considering this positive effect, in the present study, ET characterized as a Ca\(^{2+}\) regulator was used to treat 2VO model rats, and we found that ET displayed an excellent neuroprotective effect on learning and memory in 2VO rats. This positive role may be partly realized through the Ca\(^{2+}\) activated NMDAR 2B signal pathway to enhance long-term potentiation (LTP) and to decline reactive oxygen species generation and neuronal apoptosis.

In order to study the effectiveness of ET, we used a method of permanent bilateral common carotid artery occlusion to create a model of VD. After three weeks, these animals were tested by MWM task, and they showed visible spatial learning and memory deficits by longer escape latency and shorter time spent in the target quadrant. We also found severe nuclei shrinkage and neuron death in CA1 and CA3 pyramidal cells in the hippocampus. For the treatment with ET, it was shown to significantly ameliorate the cognitive impairment (Fig. 1) and reduce the neuronal loss (Fig.3).

LTP reflects a long-lasting enhancement of synaptic transmission, which is a widely accepted index of learning and memory in the hippocampus (Mans et al. 2014, Park et al. 2014). The induction of a typical form of LTP requires the Ca\(^{2+}\) influx into the dendritic spine through NMDA receptor (NMDAR), combined with calmodulin (CaM), NMDA activated CaMKII, which underwent autophosphorylation. Then a phosphorylation of CaMKII phosphorylated AMPA receptors (AMPARs), and these triggered a series of reactions to produce LTP (Lisman et al. 2002, Malenka & Nicoll 1999, Dai et al. 2007). PSD-95 is a molecular partner of NMDAR, which forms a molecular complex to contribute to the synaptic formation (Cho et al. 1992). The present results showed that the LTP was impaired in the 2VO rats, which involved in a reduced phosphorylation of CaMKII and decreased the level of both NMDAR 2B and PSD-95 protein, which was in-line with previous reported (Ge et al. 2015b, Dai et al. 2007). In turn, the neuroprotective effects of ET were observed, and the result showed that ET regulated Ca\(^{2+}\) was entering to the postsynaptic membrane by activated NMDA 2B receptor. And then the phosphorylated CaMKII enhanced the LTP. The activated NMDA 2B receptors bond to PSD-95 and promoted a synaptic transmission (Fig. 2, Fig. 5A, Fig. 6A, Fig. 6B).
Another interpretation of the positive effect of ET against ischemic injury is involved in oxidative stress. Excessive oxidative stress contributes to the progression of cognitive decline in VD (Luca et al. 2015). In this study, an elevated level of MDA and a decreased SOD were found in the hippocampus of 2VO rats. Treatment with ET diminished MDA production and enhanced SOD activities (Fig. 4). Our previous study indicates that ET inhibits excessive oxidative stress in glutamate-injured PC12 cells. Our results in the present study are consistent with that of our previous data, which demonstrates that the treatment with ET remarkably reduced oxidative stress in hippocampus.

Some reasons including excessive oxidative stress caused neuronal apoptosis play the main role for the cognitive dysfunction in VD. Bcl-2 and Bax are widely used to evaluate the level of apoptosis (Liu et al. 2015, Liu et al. 2016). If a ratio of Bcl-2/Bax is higher, the ability of anti-apoptosis will be stronger. Our current results indicated that the level of apoptosis was increased by testing the expression of Bcl-2 and Bax proteins and the TUNEL-positive neurons. A treatment with ET enhanced the ratio of Bcl-2/Bax which decreased the level of apoptosis (Fig. 5B, Fig. 6C). This was also in an agreement with our previous results of in-vitro test experiment. As in general, ET exerts a neuroprotective effect on anti-apoptosis.

In conclusion, our work is the first time to state that ET has excellent effects on learning and memory impairment in 2VO rats. Moreover, ET activates the Ca^{2+} pathway and increases phosphorylation of CaMKII, expression of NMDAR 2B and PSD95. Meanwhile the inhibition of excessive oxidative stress is related to a prevention of neuronal apoptosis in the hippocampus (Fig. 7). These information supports that the ET could be considered to be a valid neuroprotective drug to various VD’s.

**Acknowledgments and conflict of interest disclosure**

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All experiments were conducted in compliance with the ARRIVE guidelines.

**Author contributions**

Z.Y. and W.L. conceived and designed all the experiments. W.L., H.J.Y., Y.Y. performed the experiments and analyzed the data. W.L., H.J.Y., Y.Y., Y.K.C., G.G.R., Z.Y. drafted and revised the article. All authors approved the final version.

**Reference**


**Figure legends:**

**Fig. 1** Results of ET ameliorated spatial cognition in water maze task. A: Mean escape
latency in place navigation phase. B: Mean swimming speed in place navigation phase. C: Mean percentage of time in target quadrant in spatial probe phase. D: The typical swimming trace of sham group, 2VO group, 2VO+ET group. All data were expressed as mean ± S.E.M, n=6. *P<0.05, ***P<0.001 comparison between sham and 2VO groups; #P<0.05, ##P<0.01 comparison between 2VO and 2VO+ET groups.

**Fig. 2** Effects of ET on the synaptic transmission of Schaffer collateral-CA1 in vivo electrophysiological test. A: Schematic drawing of fEPSP slopes of pyramidal cells in hippocampal CA1 region. These data points were calculated from means of every 5 consecutive seconds. B: Mean fEPSP slopes between 40 and 60 min after TBS. All data are expressed as mean ± S.E.M, n=6. ***P<0.001 comparison between sham and 2VO groups; ###P<0.001 comparison between 2VO and 2VO+ET groups.

**Fig. 3** Effects of ET on morphologic changes of the CA1 and CA3 regions. H&E staining, 400×; Scale bar: 50 um. All data were expressed as mean ± S.E.M, n=3. ***P<0.001 comparison between sham and 2VO groups; ###P<0.001, #P<0.05 comparison between 2VO and 2VO+ET groups.

**Fig. 4** ET reduced oxidative stress levels in the hippocampus. A: The effect of ET on SOD activities. B: The effect of ET on MDA level. All data were expressed as mean ± S.E.M, n=3. ***P<0.001 comparison between sham and 2VO groups; ##P<0.01 comparison between 2VO and 2VO+ET groups.

**Fig. 5** ET treatment prevented apoptosis and enhanced synaptic density. A: Representative images of TUNEL-positive cells in each group, and results of image analyses using Image J for neuronal apoptosis. B: Photomicrographs of PSD95 immunostaining in the hippocampus and results of image analyses using Image J for synaptic density. All data were expressed as mean ± S.E.M, n=3. **P<0.01 comparison between sham and 2VO groups; #P<0.05 comparison between 2VO and 2VO+ET groups.
Fig. 6 Results of Western blot assay. A: Western blotting analysis indicated the effects of ET on p-CaMKII/CaMKII (A), NMDAR 2B and PSD95 (B), Bcl-2/Bax protein levels in 2VO rats. All data were expressed as mean ± S.E.M, n=3. **P<0.01, ***P<0.001 comparison between sham and 2VO groups; ##P<0.01, ###P<0.001 comparison between 2VO and 2VO+ET groups.

Fig. 7 Schematic figure illustrates the mechanism of ET rescuing cognitive deficits.

We proposed that the molecular mechanisms of the rescued learning and memory impairment by Etidronate involved in the activation of Ca\(^{2+}\) channels associated with the increased expression of NMDAR 2B and PSD95. Meanwhile it can suppress the excessive oxidative stress so as to reduce the incidence of neuronal apoptosis in hippocampus. These findings support that Etidronate can be considered as a candidate neuroprotective drug with efficacy in vascular dementia.