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Full Length Research Paper

Effects of AdR-siPTEN on learning capability, memory and extracellular signal-regulated kinase expression in hippocampus of rats with vascular dementia

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This study investigated the effects of a recombinant adenovirus, AdR-siPTEN, on learning capability, memory and extracellular signal-regulated kinase (ERK) expression in the hippocampus of rats with vascular dementia (VD). VD was introduced via permanent bilateral common carotid artery ligation (2-VO) in rats. AdR-siPTEN recombinant adenovirus and unrelated control adenovirus AdRFP were independently injected into the hippocampus of VD rats. Four weeks later, Morris water maze test was performed to detect the cognition of rats. Hematoxylin and eosin (HE) and Nissl staining were used to observe cellular morphous and neuronal damage in the hippocampus CA1 region. The mRNA and protein expression of a phosphatase and tensin homolog deleted on chromosome 10 (PTEN), phosphatidylinositol 3-kinase (PI3K), ERK and cAMP response element-binding (CREB) were detected by reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. PTEN expression was reduced apparently in hippocampus after RNAi intervation in the AdR-siPTEN group ($P<0.05$). Compared with the AdRFP group, rats in the AdR-siPTEN group had significantly shorter escape latency to the invisible platform (except on the first day of test) ($P<0.05$); and more neurons with regular shape and/or Nissl bodies in hippocampus were observed in the AdR-siPTEN group, suggesting attenuated neuronal damage and degeneration. Additionally, the expression of PI3K, ERK and CREB in hippocampus CA1 region of AdR-siPTEN treatment group was markedly higher than that in the AdRFP group ($P < 0.05$). However, compared with the normal group, the expression of PI3K, ERK and CREB was dramatically decreased in the AdRFP group ($P < 0.05$). AdR-siPTEN may improve the cognition of VD rats, attenuate neuronal damage and promote expression of ERK and CREB, leading to the protective effects on neuronal synaptic plasticity in VD rats. These results suggest that PTEN down-regulation may exert potential therapeutic effects on VD.

Key words: AdR-siPTEN, learning capability, memory, extracellular signal-regulated kinase, hippocampus, vascular dementia.

INTRODUCTION

The incidence of aging-related diseases such as arteriosclerosis, hypertension as well as various types of dementia is increasing with the increase in elderly population (Emery et al., 2005). Vascular dementia (VD) is the second most common form of dementia after Alzheimer disease (AD) and refers to a step-wise deterioration in cognitive impairment caused by loss of blood supply as a result of ischemic or hemorrhagic cerebrovascular disease or hypoxia (Kalaria et al., 2004). Following the reduction of cerebral blood flow, abnormal energy metabolism occurs after chronic cerebral ischemia which may cause neuron injury in vulnerable regions of the brain, especially in the hippocampus, resulting in cognitive

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impairment (Zarow et al., 2005). The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10), located on 10q23.3, encodes a 403-residue dual-specificity phosphatase and was identified in 1997 as the relevant gene in a region of chromosome 10 that is often absent in late-stage human tumors, especially those of brain (Li et al., 1997). PTEN has both lipid and protein phosphatase activity, and has been shown to be involved in the regulation of serine, threonine and tyrosine phosphorylation (Sarah et al., 2008), and widely distributed in the cerebral cortex, cerebellum, olfactory bulb and hippocampus (Chol et al., 2005). As a central negative regulator of phosphatidylinositol 3-kinase (PI3K) pathway involve in cell growth, proliferation and survival, PTEN has been found to regulate the development of neuron and synapse and subsequent synaptic function (Fraser et al., 2008). The extracellular signal-regulated kinase (ERK) is one of the most important members of the mitogen-activated protein kinase (MAPK) superfamily. ERK signaling pathway plays an important role in transmitting extracellular signals from cell surface to nucleus, and is involve in cell proliferation and differentiation. Recently, increasing evidence has shown that ERK signaling pathway is closely associated with learning and memory (Sheng et al., 2010). It is very well known that impairment in learning and memory is one important manifestations of VD. In addition, it has been demonstrated that the phosphatase activity of PTEN can inhibit the activation of MAPK through several pathways (Sarah et al., 2008), which is closely related to the cognitive function of dementia. In the present study, the effects of PTEN down-regulation on ERK expression, learning and memory in VD rats were investigated to explore the potential mechanisms underlying the neuronal synaptic plasticity.

MATERIALS AND METHODS

Experimental animals

A total of 30 healthy adult male Sprague-Dawley rats (specific pathogen free) weighing 250 ± 20 g were obtained from the Experimental Animal Center of Chongqiong Medical University, China. Animals were randomly divided into 3 groups (n = 10 per group): Normal, AdR-siPTEN and AdRFP group. In the AdR-siPTEN group, a recombinant adenovirus with PTEN siRNA, that was a 17-nucleotide PTEN target sequence (5'- AGCTAAAGG TGAAGATA-3') corresponding to nucleotides 1687-1703 of PTEN mRNA was selected for constructing siPTEN-red fluorescent protein (RFP) recombinant adenovirus that mediates the expression of PTEN. In the AdRFP group, a scrambled PTEN target sequence was designed to construct the control AdRFP recombinant adenovirus. The study was carried out after approval by the ethics committee of the Department of Medical Research, Chongqing Medical University, China.

Preparation of the VD model

VD was introduced via permanent bilateral common carotid artery ligation (2-VO). Adult male SD rats were anesthetized with 3.5% chloral hydrate (1 ml/g, intraperitoneally) and fixed in a supine position on a frog board. The hair in the middle neck was removed followed by sterilization with alcohol and a middle line incision of the neck was made. Bilateral common carotid arteries were then carefully separated and permanently ligated with 1-0 suture. Finally, the wound was closed (Pappas et al., 1996).

AdR-siPTEN treatment

Normal rats were fixed in a stereotaxic apparatus (USA), and 5 µl of recombinant adenovirus, AdR-siPTEN, was slowly injected into the hippocampal CA1 region (3.5 mm anterior to the bregma, 1.6 mm lateral to the middle line, 3.0 mm subdural) with a microsyringe. Seven days later, the rats were decapitated and brains were cut into sections. The red fluorescence in the hippocampus was located to determine whether the recombinant adenoviruses were accurately injected into hippocampus. Two days after injection with the recombinant adenovirus, AdR-siPTEN, VD was introduced according to the protocols aforementioned.

Behavioral test

Four weeks after surgery, the spatial learning performance of rats in each group was evaluated by Morris water maze test (Baldi et al., 2005). For descriptive data collection, the pool was divided into four quadrants: I – IV. Rats were placed into the pools (150 cm in diameter and 45 cm in height) and the water temperature was maintained at 22±2°C. A transparent platform (12 cm in diameter and 21 cm in height) was hidden in one of the quadrants. Each rat received four tests daily for 7 consecutive days. From day 1 to 6, the constant-bearing navigation test was also performed. The rats were placed into water in the order of I – IV quadrants. The escape latency (time to reach the platform within 120 s) was recorded to evaluate the spatial learning performance. If the rat failed to find the hidden platform within 120 s, we guided the rat to the platform followed by resting on the platform for 30 s, and the escape latency was recorded as 120 s.

In the present study, the escape latency on the sixth day of morris water maze test was used to evaluate the learning and memory of rats. After constant-bearing navigation test, the spatial probe test was performed to assess the retention of spatial memory on the seventh day of morris water maze test. The hidden platform was removed and each rat was put at a random starting position in the pool. The swimming trace and the number of crossing the position where the platform was set within 120 s were used to evaluate the cognitive performance (Xiong et al., 2006).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The mRNA expression of PTEN, PI3K, cAMP response element-binding (CREB) and ERK in the hippocampus of each group was determined by RT-PCR analysis. Four weeks after surgery, animals in each group were killed and hippocampus was quickly removed, and immediately frozen with dry ice. Total RNA was extracted with Trizol (TIANGEN Biotechnology) according to the manufacturer's protocol. After determining the concentration and quality of extracted RNA by spectrophotometry and 1% agarose gel electrophoresis, RNA was immediately reversely transcribed into cDNA (Tian et al., 2007). PCR amplification were carried out in a Master cycler gradient PCR instrument (Eppendorf, USA) using a protocol consisting of 94°C for 3 min in 1 cycle and denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s for a total of 35 cycles. The primers were as follows: PTEN-F: 5'-TTGAAGAC

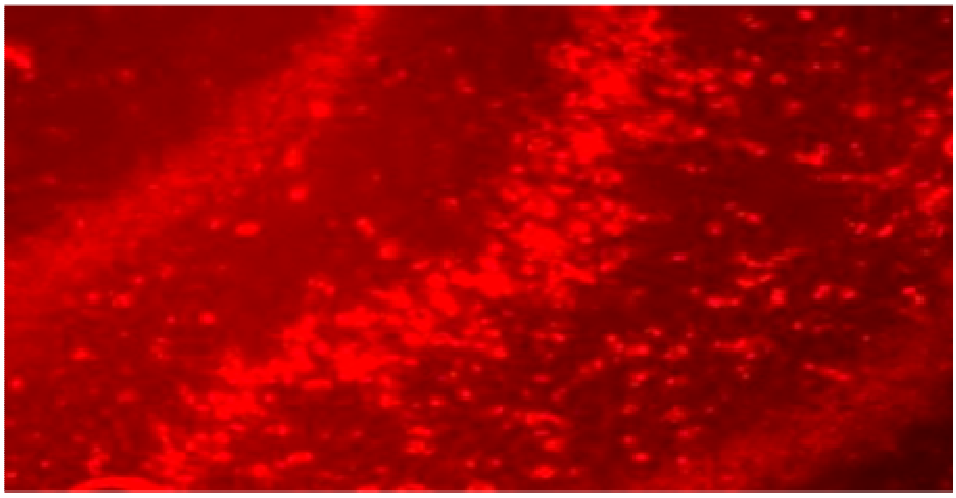


Figure 1. Red Fluorescence detected in the hippocampus zone infected with AdRsiPTEN (× 100).

CATAACCCACC-3', PTEN-R: 5'-AGTTCCGCCACTGAACAT -3', with annealing at 60℃ to obtain amplified products of 361 bp; CREB-F: 5'-TTGCCACATTAGCCCAGGTA-3', CREB-R: 5'-GTTT GGTAAATCGGGGTTGG-3', with annealing at 62℃ to obtain amplified products of 390 bp; ERk-F: 5'-TGGCTTTCTGACCGA GTATGTG -3', ERk-R: 5'-CTGTGATGCGCTTGTTTGG -3', with annealing at 62℃ to obtain amplified products of 367 bp; PI3K-F: 5'-GTCAAACCATCAAGGTCTCCG-3', PI3K-R: 5'-TGAGGGGCG TTTCACCTACTA-3', with annealing at 60℃ to give amplified products of 173 bp; GAPDH-R: 5'-TCAACGGCACAGTCAAGG-3', GAPDH-F: 5'-ACCAGTGGATGCAGGGAT-3' with annealing at 58℃ to obtain amplified products of 469 bp. The PCR products were electrophoresed with a 1% agarose gel. Images were captured and data were analyzed by Quantity one 4.6 software. The mRNA levels were calculated as ratios of intensities of specific mRNAs to intensity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Histopathology

At the end of the behavioral tests, the rats were deeply anaesthetized with 3.5% chloral hydrate, and then a catheter was intubated to the ascending aorta through the left ventricle. Transcardial perfusion with saline was performed followed by ice-cold 4% paraformaldehyde (400 ml). The brain was removed and paraffin-embedded. Coronal and serial sections (4 mm in thickness) were taken from each brain (Xiong et al., 2006). Hematoxylin and eosin (HE) and Nissl staining were performed in sections from same layers of each rat to observe the cellular morphous and neuronal damage in the hippocampus CA1 region. Under a light microscope, neurons with round- or oval-shaped nuclei and without shrinkage or edema were regarded as undamaged.

Immunohistochemistry

Animals in each group were sacrificed at 28 days post-surgery for immunohistochemistry. Immunostaining was carried out with the septum pellucidum (SP) method. The slides were prepared with same methods for HE and Nissl staining. For immuno-histochemistry, brain sections were incubated with primary antibody overnight at 4℃. The following primary antibodies (ZSGB-Bio) were used: rabbit anti-CREB (1:100), and rabbit anti-ERK (1:100). After washing with phosphate buffered saline (PBS), sections were incubated with corresponding secondary antibodies. Thereafter, sections were treated with SP staining kit (Santa Cruz Biotechnology). Then, the sections were observed under a light microscope (Olympus, Japan) connected to a computer. Representative images were obtained and mean density (IOD/area) was analyzed with Image Pro Plus 6.0 analysis system (Park et al., 2004).

Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA) and multivariate analysis of variance for the repeated measures of water maze test. The SPSS 13.0 was used for statistical analysis. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Detection of red fluorescence in hippocampus

Red fluorescence in the hippocampus after injection with recombinant adenovirus, AdR-siPTEN, was observed under a fluorescent microscope (Olympus, Japan). As shown in Figure 1, plenty of red fluorescent proteins were observed in the hippocampus demonstrating that the recombinant adenoviruses were accurately infected into the hippocampus.

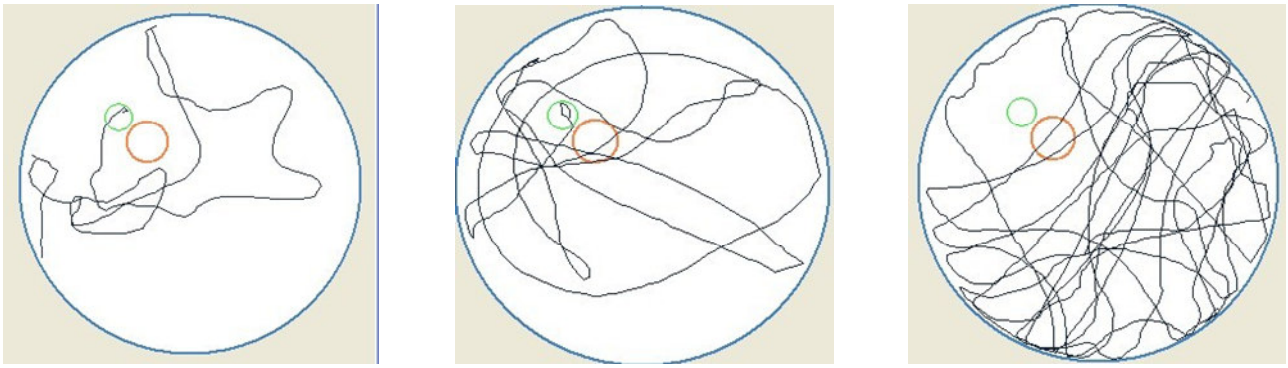
Morris water maze test

Morris water maze test was used to detect the learning and memory ability of rats. As shown in Table 1, the escape latency in AdR-siPTEN-treated group was significantly shorter than that in the AdRFP control group. Compared with normal rats, the rats in AdRFP group

Table 1. Escape latency and numbers of crossing over the platform in each group ($\bar{x}$ ±S).

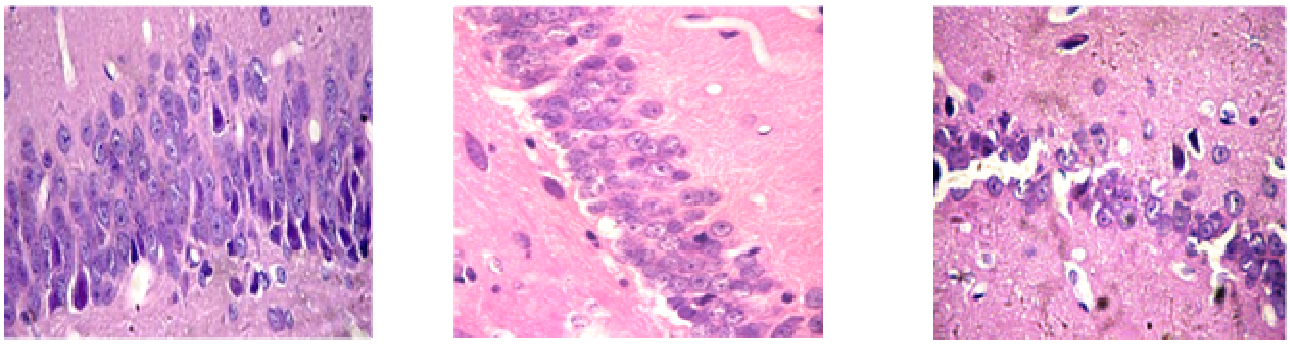
Groups	Escape latency(s)	Numbers of crossing the platform
normal	7.837±1.32	6.38±1.41
siPTEN	12.06±1.58	4.63±0.74
AdRFP	32.09±3.47*	1.63± 0.74 ##

*P < 0.05, ##P< 0.05, vs. normal group.



a. normal b. siPTEN c. AdRFP

Figure 2. Swimming traces in each group by the spatial probe test.



a. normal b. siPTEN c. AdRFP

Figure 3. Representative photographs of Pyramidal Layer of Hippocampal CA1. Area in HE Staining (×400).

spent more time in finding the platform accompanied by prolonged escape latency (P<0.05). However, the prolonged latency was significantly reduced in AdR-siPTEN-treated group compared with AdRFP group. Figure 2 shows the representative swimming traces of normal and siPTEN-treated rats determined by the spatial probe test. The traces were localized in the target zone where the platform was placed. However, in the AdRFP group, the traces were uniformly distributed in four quadrants. In addition, the numbers crossing the platform were significantly reduced in AdRFP group when compared with AdR-siPTEN treated group (P<0.05).

HE and Nissl staining

Figure 3 shows the representative photos of HE staining. Under a light microscope, a lot of cells in pyramidal layers were arranged regularly with normal morphology and integrated structures. Meanwhile, neuronal injury was observed in the hippocampal CA1 area of AdRFP treated rats. The cells in pyramidal layers were arranged irregularly, nuclei were shrunk accompanied by cell swelling and the number of normal pyramidal cells was significantly reduced. On the contrary, the abnormal morphology and decreased number of cells in hippocampal CA1 area

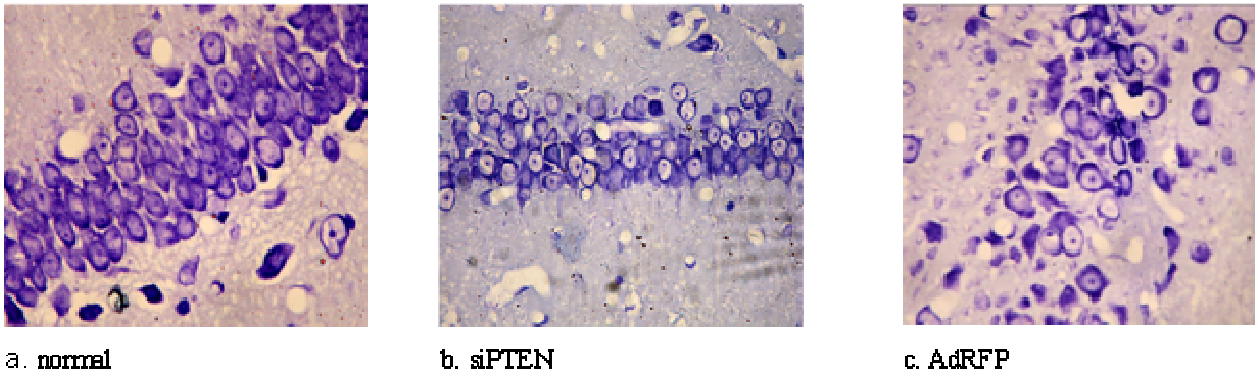


Figure 4. Nissl staining of neurons in the hippocampus of each group (×400).

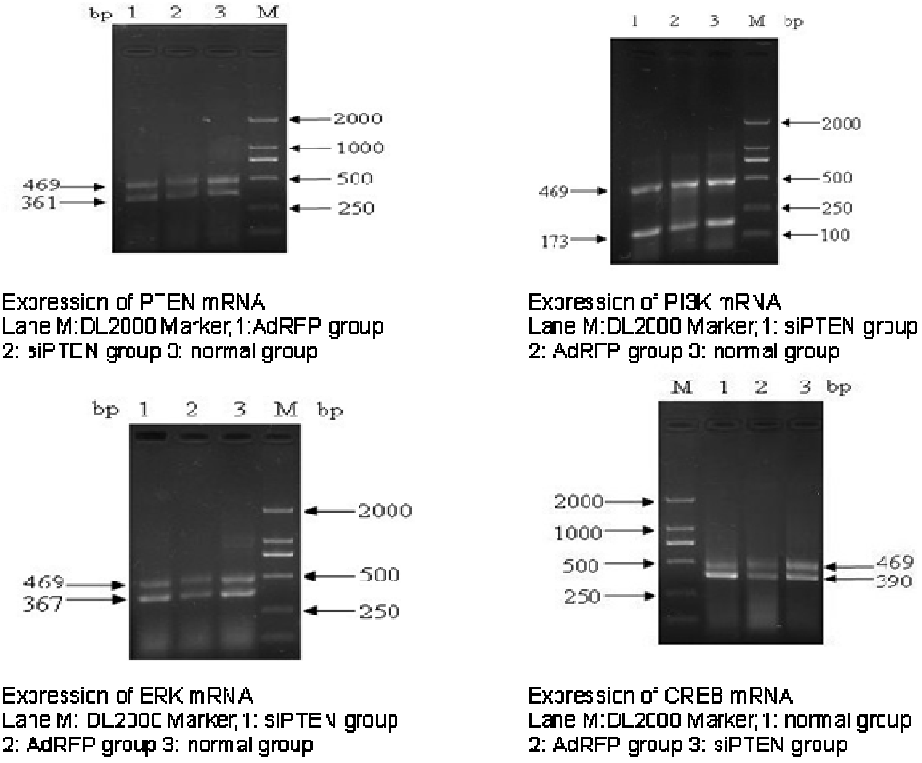


Figure 5. mRNA expression of PTEN, PI3K, CREB and ERK in each group determined by RT-PCR analysis. mRNA expression of PTEN was weakly in siPTEN group, accompanied with the converse changes of PI3K, CREB and ERK, which were weakly in AdRFP group but increased in the siPTEN group.

were markedly attenuated in siPTEN-treated group, suggesting PTEN down-regulation prevented the neuronal injury caused by chronic cerebral ischemia. As shown in Figure 4, in the normal group, the Nissl body was obviously observed in the Nissl staining. However, a majority of neurons in the AdRFP group underwent peripheral edema with blue cytoplasm, and the cell body collapsed or shrunk. Meanwhile, the Nissl body in the cells formed into granules or even vanished. The collapse and shrinkage of cell body were dramatically improved in the AdR-siPTEN-treated group, and more Nissl body was observed, indicating improved neuronal damage.

mRNA expression of PI3K, CREB and ERK

The mRNA expressions of PTEN, PI3K, CREB and ERK in each group were determined by RT-PCR. As shown in Figure 5, compared with AdRFP group, the mRNA expression of PTEN was markedly attenuated in the

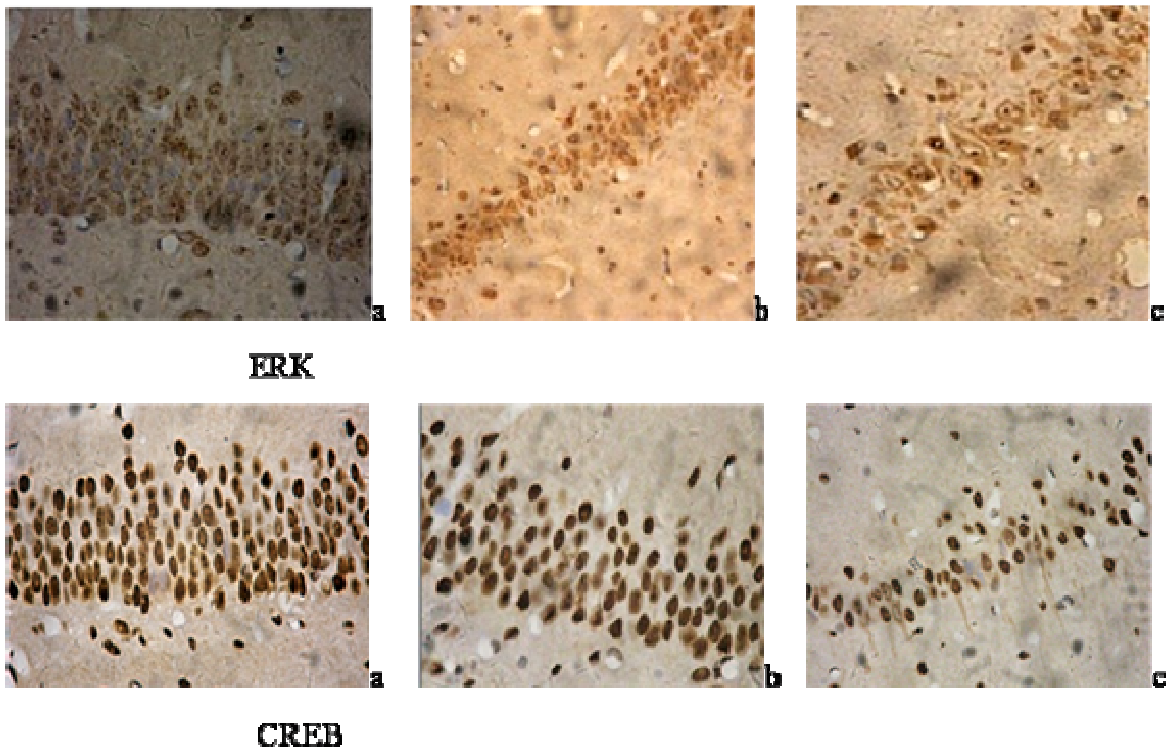


Figure 6. Protein expression of ERK and CREB in hippocampus CA1 region of each group determined by immunohistochemistry (×400). a: Normal group; b: AdR-siPTEN group; c: AdRFP group.

AdR-siPTEN group (P<0.05). The mRNA expressions of these three genes were increased in the AdR-siPTEN group accompanied by decrease in PTEN expression. In addition, the mRNA expressions of PI3K, CREB and ERK in AdRFP group were significantly lower than those in the normal roup and AdR-siPTEN group (P<0.05). These results indicated that down-regulation of PTEN might be involved in and likely contribute to the increased expression of PI3K, CREB and ERK.

Immunohistochemistry

Immunohistochemistry showed CREB and ERK positive cells in the normal group. The analysis of immunohistological findings in the AdR-siPTEN and AdRFP group is shown in Figure 6. The positive cells were stained brown-yellow. The expressions of CREB and ERK were markedly increased in normal hippocampus CA1 area. However, AdR-siPTEN treatment improved the activation of CREB and ERK in the hippocampus and increased the number of positive neurons for CREB or ERK, as compared to the AdRFP group (P<0.05). Results of image analysis of immunohistological findings are shown in Figure 7. The mean density of CREB and ERK-positive reaction product in AdRFP group was significantly lower than that in the normal group, while the AdR-siPTEN treatment group had a higher mean density as compared to the AdRFP group (P<0.05).

DISCUSSION

In the present study, the major findings were that PTEN down-regulation significantly elevated the decreased expression of PI3K/ERK/CREB (cAMP response element-binding protein) in the hippcampal lesions. A potent protection against neuronal injury caused by chronic cerebral ischemia was shown, leading to improvement in the neurological dysfunction and symptoms of VD in rats.

VD is usually thought to be caused by various cerebrovascular injury and numerous studies have demonstrated that remains the second common diseases among age-related dementia (Yanpallewar et al., 2004; Zhao et al., 2005; Borlongan et al., 2005). It has been reported that cerebral ischemia can develop cognitive deficit and neuronal damage. VD patients are usually found to have cerebral ischemia and cognitive deficit in clinical trials with large sample size. In the present study, the permanent occlusion of bilateral common carotid arteries was introduced, which can mimic the VD in humans. However, the pathogenesis of VD is very complex and still not completely clarified. In recent years, increasing studies have shown that changes in synaptic plasticity play an important role in the pathogenesis of VD. Synaptic damage is observed at the early stage of

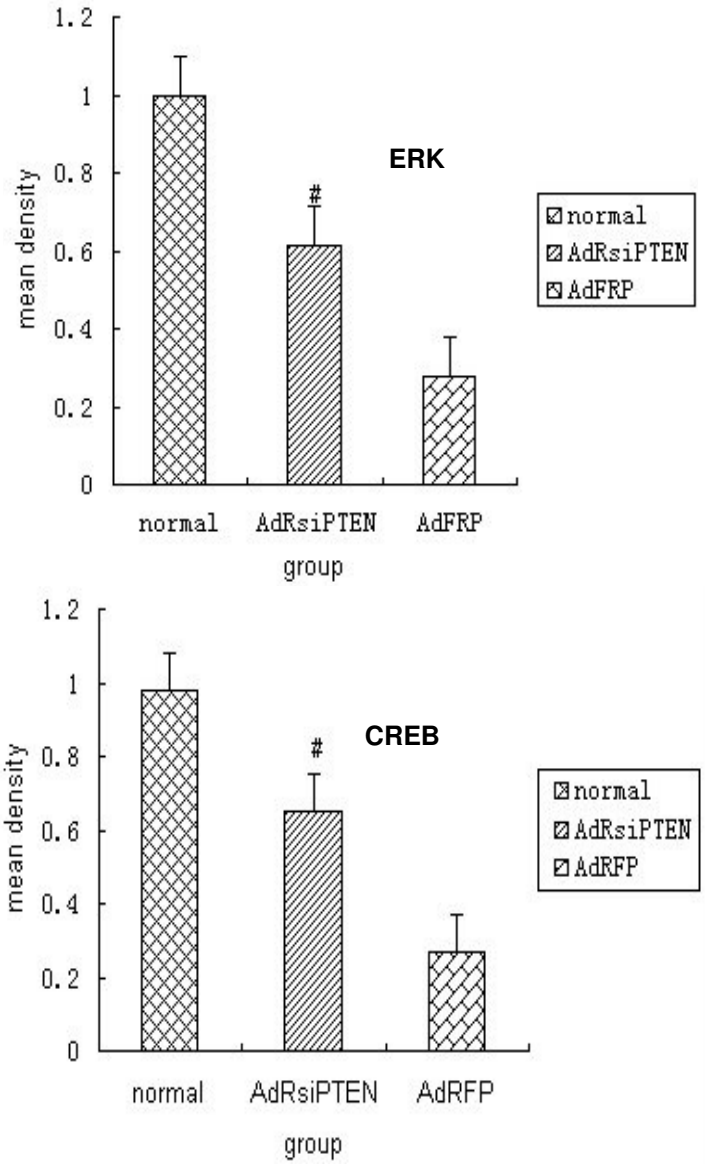


Figure 7. Mean density of ERK and CREB in each group. # P < 0.05, vs.AdRFP group.

VD, and closely associated with cognitive deficit, but the exact mechanism is poorly understood. As a negative regulator of PI3K pathway, PTEN is involved in the development of neuron and synapse and subsequent synaptic function (Fraser et al., 2008). The PI3K/Akt signaling pathway can regulate the expression of ERK and CREB, which is closely related to the cognitive function in VD patients. Our results showed that down-regulation of PTEN definitely elevated the decreased expression of PI3K/ ERK/CREB in the hippocampus of VD rats, participating in the protection of synaptic plasticity.

The present study focused on the relationship between PTEN and PI3K, ERK, CREB in the ischemic brain. PTEN is known to negatively regulate several cellular functions, including cell cycle progression, cell migration, and survival by suppressing PI3K/Akt activation via dephosphorylating the phosphatidylinositol (3,4,5)-triphosphate(PIP3) to an inactive state of phosphatidylinositol (4,5)-diphosphate(PIP2). In recent years, studies have focused on the relationship between PTEN and cerebral ischemia. It has been reported that PTEN plays an important role in neuroprotection. Jeong et al. (2004) found that cilostazol could ameliorate the neuronal damage by down-regulating PTEN phosphorylation with resultant increase in the Akt and CREB phosphorylation and Bcl-2 expression to suppress apoptotic cell death which contributed to the improvement in the neurological deterioration. Furthermore, studies have shown that inhibitors of PTEN-pic were also involved in the activation of PI3K/Akt signaling pathway through inhibiting PTEN

expression which resulted in the protection of ischemic neurons (Xiaohui et al., 2007). Additionally, besides the PI3K/Akt signaling pathways, ERK signaling pathway also plays an important part in the biological functions of PTEN. Studies have indicated that PTEN can inhibit ERK activation through its phosphatase activity. Thus, it is speculated that PTEN confers regulatory effects through ERK signaling pathway. There is a negative correlation between PTEN and ERK (Qiuxue et al., 2006). In the present study, after infection with recombinant adenovirus siPTEN, the PTEN expression in the hippocampus was significantly declined accompanied by profoundly increased expressions of PI3K /ERK/CREB which further down-regulated PTEN expression. These results were consistent with previous studies.

The ERK is one of the important members of the MAPK superfamily. It has been reported that the ERK signaling pathway is involve in cell growth, proliferation and differentiation. In recent years, accumulating evidence has shown that ERK signaling pathway was closely associated with learning and memory (Giovannini, 2006; Sweatt, 2004; Ji et al., 2009; Shaywitz and Greenbe, 1999). A study showed that MEK inhibitor (PD98059) could inhibit hippocampal long-term potentiation (LTP), which is generally accepted as one of the cellular mechanisms involved in learning and memory (Sheng et al., 2010). Behavioral studies showed the activation of ERK in the hippocampus after water maze training in mice. Furthermore, recent investigation also demonstrated Ras/ERK pathways were involved in human cognition. ERK kinase inhibitors could block the sustained phosphorylation of CREB residue Ser133 and affect the CREB-dependent transcription, suggesting ERK plays a role in regulating the CREB activity. That is to say, through the regulation of certain nuclear transcription factors, such as CREB, ERK plays a regulatory role in the LTP, as well as learning and memory. In our study, the results of both RT-PCR and immunohistochemistry demonstrated the expression of ERK and CREB was decreased in the hippocampus CA1 region of VD rats, which was obviously reversed in the siPTEN-treated rats with VD accompanied by a lower expression of PTEN. The results above suggested PTEN down-regulation definitely increased the expression of ERK and CREB, thereby participating in the neuronal synaptic plasticity and the improvement of learning and memory of VD rats. There might be several pathways implicated in the protective effects of down-regulated PTEN expression. 1) As the inhibition of ERK by PTEN through Ras or calcium signaling pathway was inhibited after PTEN down-regulation, the expression and activation of ERK were increased accompanied by improvement of learning and memory; 2) PTEN down-regulation suppresses apoptotic cell death and promotes cell growth via activating PI3K/Akt signaling pathway with resultant increase in Akt and CREB phosphorylation and increased Bcl-2 expression resulting in amelioration of neuronal damage. In addition, CREB is accepted as a key factor in cell survival and involves in regulating regeneration, survival and repair of neurons after ischemic injury. Therefore, increased CREB activity leads to the protection of learning and memory as well as synaptic plasticity. Additionally, the activation of ERK can regulate synaptic proteins and promote formation of new dendritic spines. Increased ERK level affects the phosphorylation of nuclear transcription and translation factors related to LTP suggesting synaptic plasticity, and plays positive regulatory role in the induction and maintenance of LTP, resulting in the improvement of learning and memory in VD rats.

In conclusion, the present study demonstrated that PTEN down-regulation via AdR-siPTEN improved the cognitive impairment caused by chronic cerebral ischemia, attenuated neuronal injury and protected synaptic plasticity. These findings provide a new target for the prevention and treatment of VD. However, the specific mechanisms still remain unclear, and further studies are needed.

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Abbreviations

VD, Vascular dementia; **AD,** Alzheimer disease; **PTEN,** phosphatase and tensin homolog deleted on chromosome 10; **PI3K,** phosphatidylinositol 3-kinase; **ERK,** extracellular signal-regulated kinase; **MAPK,** mitogen-activated protein kinase; **RT-PCR,** reverse transcription polymerase chain reaction; **RFP,** red fluorescent protein; **CREB,** cAMP response element-binding; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **HE,** Hematoxylin and eosin; **SP,** septum pellucidum.

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