Effect of adenovirus-mediated overexpression of PTEN on brain oxidative damage and neuroinflammation in a rat kindling model of epilepsy

Zhi-Sheng Wu, Wen-Li Huang, Shu-Jie Gong

Department of Neurology, The First Affiliated Hospital of Quanzhou, Fujian Medical University, Quanzhou, Fujian 362000, China.

Abstract
Background: Epilepsy is a chronic and severe neurological disorder. Phosphatase and tensin homolog deleted on chromosome ten (PTEN)-deficient mice exhibit learning and memory deficits and spontaneous epilepsy. The aim of this study was to investigate the role of PTEN in brain oxidative damage and neuroinflammation in a rat model of epilepsy.

Methods: An adenovirus (Ad)-PTEN vector was constructed, and status epilepticus (SE) was induced in 41 model rats using lithium chloride-pilocarpine. Thirty-six SE rats were then allocated into the Ad-PTEN, Ad-LacZ, and SE groups, those were administered intracerebroventricular injections of Ad-PTEN, Ad-enhanced green fluorescent protein, and phosphate buffer saline, respectively. The normal group was comprised of healthy Sprague-Dawley rats. Nissl staining was conducted to evaluate neuronal damage, and immunohistochemistry was conducted to observe the morphology of cells in the hippocampal CA1 region and the distribution of ionized calcium-binding adaptor molecule 1 (Iba1) and ED1 (rat homologue of human CD68). Levels of apoptosis-related proteins, inflammatory-related factors, and oxidative stress-related markers (reactive oxygen species [ROS], glutathione [GSH], superoxide dismutase [SOD], and malondialdehyde [MDA]) were measured. Comparisons between multiple groups were conducted using one-way analysis of variance (ANOVA), and pairwise comparisons after ANOVA were conducted using the Tukey multiple comparisons test.

Results: After SE induction, PTEN expression in the rat brain exhibited a four-fold decrease \((P = 0.000)\) and the expression of both Iba1 and ED1 increased. Furthermore, significant neuronal loss, oxidative damage, and neuroinflammation were observed in the SE rat brain. After intracerebroventricular injection of Ad-PTEN, PTEN expression exhibited a three-fold increase \((P = 0.003)\), and the expression of both Iba1 and ED1 decreased. Additionally, neurons were restored and neuronal apoptosis was inhibited. Furthermore, ROS and MDA levels decreased, GSH level and SOD activity increased, and neuroinflammation was reduced.

Conclusion: Our study demonstrated that brain oxidative damage and neuroinflammation in SE rats were ameliorated by intracerebroventricular injection of Ad-PTEN.

Keywords: Epilepsy; Status epilepticus; Brain injuries; Oxidative stress; Neurons; Inflammation

Introduction
Epilepsy is a chronic neurological disorder which is characterized by recurrent unprovoked seizures and is caused by overstimulation and super-synchrony of brain neurons.\[^1,2\] Status epilepticus (SE) is a neurological disorder which is characterized by prolonged epileptic activity or frequent seizures without return to baseline.\[^3\] SE is considered a medical emergency. Prolonged SE involves muscle contractile dysfunction and elevates serum creatine kinase levels, increasing energy and oxygen consumption, inducing metabolic acidosis, reducing adenosine triphosphate levels, and ultimately contributing to renal failure.\[^4\] In spite of many developments over the past years, nearly 30% of all epilepsy cases remain refractory to medication and seizures persist even after the administration of optimum drug treatment.\[^5\] Therefore, the development of novel treatments for epilepsy is of great importance.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) was initially identified as a tumor suppressor and is known to play pivotal roles in cell proliferation, migration, and stem cell renewal.\[^6\] PTEN deficiency can lead to serious brain dysfunction, including ataxia, seizures, and brain enlargement.\[^7\] A previous study...
reported the involvement of PTEN in the activation of an inflammatory response in rat hippocampal neurons.\[10\]

Furthermore, a recent study demonstrated that the microRNA-21-5p/PTEN/mammalian target of rapamycin (mTOR) signaling pathway may be a potential target for the treatment of SE-associated damage.\[8\] Therefore, we hypothesized that PTEN may be involved in SE. The aim of this study was to investigate the role of PTEN in brain oxidative damage and neuroinflammation in a rat model of epilepsy.

**Methods**

**Ethical approval**

This study was approved and supervised by the Ethics Committee of the First Affiliated Hospital of Quanzhou (No. FJMU IACUC20181124-06). All procedures conducted in this study were in accordance with our institutional guidelines and complied with the international ethics for animal use. Significant efforts were made to minimize the number of animals used and their suffering.

**Construction of adenovirus (Ad) vectors**

The coding sequence of PTEN (ID: 5728) was downloaded from the National Center for Biotechnology Information and the corresponding primer was designed. After cloning PTEN from human serum DNA, the gene was recombined with an enhanced green fluorescent protein (EGFP)-expressing Ad vector (Beijing FivePlus Molecular Medicine Institute Co., Ltd, Beijing, China). Successful construction of the vector was confirmed by sequencing and restriction enzyme digestion. The vector was named Ad-PTEN and used in subsequent experiments.

**Model establishment and rat grouping**

Fifty-three healthy Sprague-Dawley rats (200–250 g; Laboratory Animal Center of Xi’an Jiaotong University Healthy Science Center, Xi’an, Shaanxi, China, Certificate No. SYXX (shan) 2015-002) were used in this study. The normal group comprised 12 rats, and 41 rats were randomly selected for the SE model establishment. All rats were provided with free access to food and water. For SE induction, the rats were intraperitoneally injected with lithium chloride (127 mg/kg). Eighteen hours later, atropine sulfate (1 mg/kg) was also injected intraperitoneally to reduce the peripheral cholinergic effect. Thirty minutes later, pilocarpine (20 mg/kg for the first time, 10 mg/kg for the second) was injected intraperitoneally twice, with a 30-min interval between injections. After 1 h of epilepsy, diazepam (10 mg/kg) (all agents from Sigma-Aldrich Chemical Company, Shanghai, China) was injected intraperitoneally to relieve convulsions. If the epileptic seizures could not be alleviated, diazepam was injected once or twice more until the seizures were relieved. The Racine scale was used to evaluate convulsions. The rats whose epileptic seizures reached grade IV and above, and who were in a good state after seizure alleviation were considered successful SE models. Ultimately, 36 of the 41 model rats met the eligibility criteria for inclusion in subsequent experiments.

Thirty-six rat kindling models of epilepsy were numbered according to body weight, and assigned into SE (rats were injected with 10-μL phosphate buffer saline [PBS]), Ad-PTEN (rats were injected with 1 × 10^7 pfu/10 μL purified solution of recombinant Ad-PTEN), and Ad-LacZ groups (rats were injected with 1 × 10^7 pfu/10 μL Ad-EGFP). This was performed according to a random number table, and 12 rats were allocated to each group. After 3 days of treatment, rats in each group were sacrificed and their brains were removed for subsequent experiments. The brains of six rats from the SE and normal groups were removed, fixed in paraformaldehyde, embedded in paraffin, and sliced into transverse sections for histological staining. The remaining six rats in each group were used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Western blotting analysis, and oxidative stress tests.

**Nissl staining**

Hippocampal tissue 2.64 to 5.40 mm posterior to the bregma was selected, de-waxed, hydrated, and stained with Nissl staining solution (Shanghai Beyotime Biotechnology Co., Ltd, Shanghai, China) for 10 min. After ethanol (Beyotime) dehydration and washing with distilled water, sections were cleared with xylene (Beyotime), sealed with neutral gum (Beyotime), and observed under the microscope (Olympus Optical Co., Ltd, Tokyo, Japan). The number of Nissl-stained cells was then counted.

**Immunohistochemistry**

Hippocampal tissue 2.64 to 5.40 mm posterior to the bregma was selected, de-waxed, hydrated, and added to 500-mL citric acid-sodium citrate solution (92–98°C, 10 g/L; Beyotime) for 15 min. After incubation with hydrogen peroxide (0.882 mol/L; Beyotime) at 20 to 25°C for 10 min, sections were washed with PBS three times, sealed with a blocking buffer containing 100 mL/L bovine serum, and incubated at 20 to 25°C for 1 h. Following serum removal, sections were incubated with primary antibodies overnight at 4°C, and then incubated with a secondary antibody at 37°C for 30 min after three PBS washes. The antibodies used were anti-ionized calcium-binding adapter molecule 1 (Iba1) (1:200, ab48004), anti-ED1 (rat homolog of human CD68 [PMID30630985]) (1:100, ab31630), and anti-immunoglobulin G (1:200, ab97035) (all from Abcam, Cambridge, MA, USA). Afterward, sections were stained with 2,4-diaminobutyric acid, rinsed with distilled water, re-stained, dehydrated, cleared, and sealed with neutral gum. After being dried, sections were observed and photographed under the microscope (Olympus Optical Co., Ltd.). Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA) was used for image analysis.

**RT-qPCR**

TRizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from isolated hippocampal tissue, and the extracted high-quality RNA was verified using ultraviolet analysis and formaldehyde denaturation electrophoresis. PCR primers were designed and synthesized.
Table 1: Primer sequences for RT-qPCR.

| Genes   | Sequences                        |
|---------|----------------------------------|
| PTEN    | F: 5’-CAATGACAGCCATCATCAAAGAG-3’  |
|         | R: 5’-GCTCAGACTTTTGTAATTGTG-3’   |
| GAPDH   | F: 5’-TGGGTGTAACCCTGAGAA-3’      |
|         | R: 5’-GTGCCTGTTGAAGTCA-3’        |

F: Forward; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; R: Reverse; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

Western blotting analysis
Cellular proteins in the rat hippocampus were extracted, and protein concentrations were determined according to the instructions of the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The extracted proteins were added to loading buffer, boiled at 95°C for 10 min, loaded into each well (30 μg of each protein), and separated by 10% w/v sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the voltage alternating between 80 and 120 V. The proteins were transferred to a polyvinylidene fluoride membrane by wet transformation at 100 mv for 10 min, loaded into each well (30 μg of each protein), and incubated overnight with primary antibodies at 4°C. Afterward, the membrane was rinsed three times (5 min/time) with Tris-buffered saline with Tween. The membrane was then incubated with a horseradish peroxidase-labeled secondary antibody (ZSGB-Bio Co., Ltd, Beijing, China) at 20 to 25°C for 1 h. The antibodies used were as follows: anti-PTEN (1:1000, ab31392), anti-interleukin (IL)-1β (1:1000, ab9722), anti-β-actin (1:1500, ab8227), anti-tumor necrosis factor-α (TNF-α) (1:1000, ab6671), anti-Bcl-2-associated X (Bax) (1:1000, ab53154), anti-B-cell lymphoma-2 (Bcl-2) (1:1500, ab196495), and anti-nuclear factor-kappa B (NF-κB) (1:1000, ab32360) (all from Abcam Inc.). The membrane was then washed and developed using an enhanced chemiluminescence reagent, and bands were visualized using the Gel Dol EZ imager developed using an enhanced chemiluminescence reagent, Abcam Inc.). The membrane was then washed and developed using an enhanced chemiluminescence reagent, and bands were visualized using the Gel Dol EZ imager (Bio-Rad Laboratories, Hercules, CA, USA). The target bands were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA) for gray value analysis.

Measurement of oxidative stress-related factors
To determine reactive oxygen species (ROS) levels, hippocampal tissues were cut into pieces and neurons were isolated using an ophthalmic nipper. The cells were collected after centrifugation at 500 × g for 10 min, washed with PBS, and incubated with dichlorofluorescein diacetate buffer (MedChemExpress, Princeton, NJ, USA) at 37°C for 30 min. Fluorescence intensity was measured using a microplate reader (Thermo-Max, Molecular Devices, Sunnyvale, CA, USA) with excitation/emission at 493/522 nm. The results were expressed as the ratio of ROS content to that of the normal group.

Hippocampal neurons were collected using the aforementioned procedures. Cells were subjected to different treatments according to the instructions of the total glutathione (GSH) detection kit, total superoxide dismutase (SOD) activity detection kit, and malondialdehyde (MDA) detection kit (all from Beyotime). Using a microplate reader, the absorbance at 410 nm was measured to calculate the total GSH content. The absorbance at 560 nm was measured to calculate SOD activity. The absorbance at 532 nm was measured to calculate MDA content.

Statistical analysis
SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for data analysis. The Kolmogorov-Smirnov test was employed to check whether the data were normally distributed. The results are described as mean ± standard deviation. Comparisons between multiple groups were analyzed by one-way analysis of variance (ANOVA), and pairwise comparisons after ANOVA were conducted using the Tukey multiple comparisons test. P values were obtained from two-tailed tests, and a P < 0.05 was considered statistically significant.

Results
Ad-PTEN increases PTEN expression in brain tissue of SE rats
The RT-qPCR and Western blotting analysis results showed that PTEN expression exhibited a four-fold decrease after SE induction (P = 0.000). After intracerebroventricular injection of Ad-PTEN, PTEN expression exhibited a three-fold increase (P = 0.003). There was no significant difference in PTEN expression between the Ad-LacZ and SE groups (P = 0.978) [Figure 1A and 1B]. Thus, these results suggested that PTEN may play an important role in epilepsy.

Ad-PTEN inhibits microglial activation in brain tissue of SE rats
The immunohistochemistry results are presented in Figure 2A. The results of Iba1 staining (a marker of microglia) demonstrated that after SE induction in rats, the size of microglial cell bodies was significantly increased, and the area of Iba-positive cells was signiﬁcantly larger than that of normal rats. The results of ED1 staining demonstrated that ED1 expression was very minimal in the normal rats, but increased signiﬁcantly in the hippocampus of SE rats. After Ad-PTEN injection, Iba1 and ED1 expression in the hippocampus of SE rats signiﬁcantly decreased. Immunofluorescence staining showed that ED1 expression co-localized with Iba1-positive cells in the normal rats, and these cells were primarily expressed in the
After Ad-PTEN injection, few cells co-expressed ED1 and Iba1 except for that the size of microglial cell bodies was reduced [Figure 2B]. There were no notable differences in the immunohistochemistry or immunofluorescence results between the Ad-LacZ and SE groups.

**Ad-PTEN decreases neuronal loss and apoptosis in SE rats**

The results of Nissl staining demonstrated a significant loss of neurons in the hippocampus of SE rats. Neurons could be restored after intracerebroventricular injection of Ad-PTEN [Figure 3A]. Bcl-2 and Bax are typical apoptosis-related proteins which elicit opposite effects: Bcl-2 inhibits apoptosis, while Bax promotes apoptosis. The hippocampal expression of Bcl-2 was significantly lower in SE rats than in normal rats \((P = 0.000)\). In contrast, hippocampal Bax expression was significantly higher in SE rats than in normal rats \((P = 0.000)\). Ad-PTEN injection attenuated the SE-induced reduction in Bcl-2 expression and increase in Bax expression \((P < 0.001)\). There were no notable differences between the Ad-LacZ and SE groups with respect to Bcl-2 or Bax expression \((P = 0.416, 0.330,\) respectively) [Figure 3B].

**Ad-PTEN improves oxidative damage in brain of SE rats**

Figure 4A shows that the fluorescence intensity of ROS in the hippocampus of SE rats was nearly three times higher than that of normal rats. The fluorescence intensity of ROS in the SE rat hippocampus was significantly decreased after Ad-PTEN injection \((P = 0.000)\). Compared with normal rats, the GSH content and SOD activity in the SE rat hippocampus were significantly decreased \((P = 0.000)\). The MDA content of the SE rat hippocampus was significantly increased \((P = 0.000)\). These alterations in GSH, SOD, and MDA were reversed by Ad-PTEN injection. There were no significant differences between the Ad-LacZ and SE groups with respect to ROS, GSH, or MDA content, or SOD activity \((P = 0.966, 0.988, 0.537, 0.985,\) respectively) [Figures 4B–D]. These results suggested that recombinant Ad-PTEN could effectively improve oxidative damage in the SE rat brain.

**Ad-PTEN mitigates neuroinflammation in SE rats**

Western blotting analysis was conducted to detect the levels of the inflammatory-related factors TNF-α, NF-κB, and IL-1β in the rat hippocampus. The results demonstrated that the levels of TNF-α, NF-κB, and IL-1β were significantly higher in the hippocampus of SE rats compared to normal rats \((P = 0.000)\). After Ad-PTEN injection, the levels of the above factors were significantly decreased \((P = 0.000)\). There were no significant differences between the Ad-LacZ and SE groups with respect to the levels of these inflammatory-related factors \((P = 0.416, 0.999,\) and 0.752, respectively) [Figure 5A–D]. Therefore, we concluded that recombinant Ad-PTEN could significantly improve neuroinflammation in SE rats.
Epilepsy is one of the world’s most serious neurological disorders and is characterized by initial damage due to stroke, brain infection, traumatic brain injury, or thermal seizures.\(^{10}\) Nearly 30% of all epilepsy cases remain refractory to medication, and drug and surgical treatment often lead to serious side effects.\(^{11}\) A previous study demonstrated that PTEN is involved in excitotoxic...
damage, neuronal death, and inflammation in epilepsy.\[12]\nTherefore, the present study investigated the effects of PTEN overexpression in a rat model of epilepsy. Of crucial importance, our study demonstrated that recombinant Ad-PTEN ameliorated brain oxidative damage and neuroinflammation in rats with epilepsy.

First, we observed low hippocampal PTEN expression in SE rats. This expression was significantly increased after Ad-PTEN injection. It has been observed that epilepsy patients with PTEN mutations exhibited spontaneous seizures, and PTEN deficiency increased seizure susceptibility in mice.\[13]\nLaSarge et al\[14]\n demonstrated that the deletion of PTEN from hippocampal dentate granule cells resulted in mTOR hyperactivation and facilitated the quick occurrence of spontaneous seizures. PTEN deletion has also been associated with a remarkably shorter recurrence time and poor outcome of prostate cancer, and a lower 5-year overall survival in patients with pancreatic neuroendocrine tumors.\[15,16]\nSecond, we found that Ad-PTEN decreased the numbers of Iba1- and ED1-positive cells, representing the inhibition of microglial activation. In a study of patients with Rasmussen’s encephalitis, a condition characterized by acute seizure activity, Iba1 was found to be an effective marker of microglial activation.\[17]\nFurthermore, ED1 has been recognized as a marker of macrophages and microglial activation.\[18]\nSE-induced microglial activation has been associated with hippocampal neuronal loss and segmental neurodegeneration, and has been shown to have a critical role in the pathogenesis of mesial temporal lobe epilepsy.\[19]\nIn this study, after recombinant Ad-PTEN injection, TNF-α, NF-κB, and IL-1β levels were significantly decreased. According to Vezzani et al.\[20]\nprolonged seizures induce the release of inflammatory mediators and
consequently increase the severity of subsequent seizures and epileptogenesis. Additionally, activated microglia may promote neuroinflammation and neurodegenerative process by releasing IL-1β and TNF-α, possibly resulting in neuronal damage and death.\[22\] Consistent with this, Yang et al.\[23\] reported that IL-1β and TNF-α were upregulated in certain brain regions of patients with temporal lobe epilepsy. Thus, the inhibition of inflammatory factors may provide neuroprotective effects in epilepsy.\[10\] Ad-PTEN was also reported to reduce TNF-α and IL-1β levels and inhibit NF-κB activation in neoinnatum formation.\[24\] Therefore, we concluded that recombinant Ad-PTEN could effectively improve neuroinflammation in epileptic rats.

Furthermore, our data revealed that recombinant Ad-PTEN injection attenuated the SE-induced reduction in Bcl-2 expression and increase in Bax expression. In hyperthermic rats with simple febrile seizures, hippocampal Bcl-2 expression was significantly decreased but pro-apoptotic Bax expression was significantly increased.\[25\] Significantly decreased Bax mRNA levels were observed in breast cancer HEK293 cells overexpressing PTEN ATP-binding mutations.\[26\] In our study, after recombinant Ad-PTEN injection, the hippocampal GSH content and SOD activity were significantly decreased, while the ROS and MDA contents were decreased. A previous study found that PTEN deletion resulted in increased levels of ROS and oxidative damage and reduced SOD activity in mouse embryonic fibroblasts.\[27\] Furthermore, repeated seizures led to increased ROS production and oxidative damage, potentially resulting in significant brain damage.\[28\] In a study into skin aging, PTEN downregulation was determined to initiate ROS release, and Ad-PTEN injection-induced PTEN overexpression reduced ROS level.\[29\] GSH has been identified as a potent scavenger of free radical species and ROS, and neurodegenerative diseases have been associated with GSH depletion.\[30\] Furthermore, a reduced level of GSH correlated with an increased level of oxidative stress.\[31\] Epilepsy-related neuronal loss may diminish glial glutamate transporters, potentially enhancing extra-cellular glutamate levels and causing further neuronal loss.\[32\] MDA, a key marker of oxidative stress, was generated in the oxidative degradation of polyunsaturated lipids.\[33\] In addition, ROS induced lipid peroxidation and MDA production, in turn contributing to neurotoxicity and cell death.\[34\] Increased MDA levels have been associated with an earlier age of epilepsy onset.\[35\] PTEN overexpression enhanced the enzyme activities of SOD and GSH peroxidase in non-small cell lung cancer cells.\[36\] From our results, we demonstrated that Ad-PTEN repressed apoptosis and oxidative damage in epileptic rats.

In summary, our results demonstrated that recombinant Ad-PTEN ameliorated brain oxidative damage and neuroinflammation in SE rats. Therefore, this study provided evidence that Ad-PTEN could be useful in the development of novel therapeutic strategies for epilepsy. To explore the therapeutic potential of PTEN in epilepsy, further studies are required to evaluate its clinical efficacy and safety.

Conflicts of interest
None.

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