

Genistein Protects Epilepsy-Induced Brain Injury Through Regulating The JAK2/STAT3 and Keap1/Nrf2 Signaling Pathways in The Developing Rats

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Abstract

Background: Epilepsy is a common chronic neurological disease caused by the over-synchronization of neurons that lead to brain dysfunction. Recurrent seizures or status epilepticus can cause irreversible brain damage. The JAK2-STAT3 signal transduction pathway is stimulated by cytokines and involved in various pathological processes including inflammation, apoptosis and immune regulation in central system diseases. Keap1/Nrf2 is an important anti-oxidative stress pathway, which can reduce the toxic effects of oxygen free radicals and endogenous toxins on neurons. Genistein (Gen) can modulate inflammation and neuronal apoptosis, and may thereby have antiepileptic effects. This study aimed to explore the regulation of Genistein on JAK2/STAT3 and Keap1/Nrf2 signaling pathway and the protective effects on brain injury after epilepsy.

Methods: Pentylentetrazole (PTZ) was used to induce epilepsy in developing rats and Genistein was used for pretreatment of epilepsy. The seizure latency, grade scores and duration of the first generalized tonic-clonic seizure (GTCs) were recorded. Hippocampus tissue was sampled at 24 hours post-epilepsy. Immunofluorescence staining was used to observe the number of mature neurons, activated microglia and astrocytes in the hippocampal CA1 region. Western blot and qRT-PCR were used to determine the protein and mRNA levels of p-JAK2, p-STAT3, TNF- α , IL-1 β , Keap1, Nrf2, HO-1, NQO1, caspase3, Bax and Bcl2 in the hippocampus.

Results: Immunofluorescence showed that the number of neurons significantly decreased, and activated microglia and astrocytes significantly increased after epilepsy; Western blot and q-PCR showed that the expressions of p-JAK2, p-STAT3, TNF- α , IL-1 β , Keap1, caspase3 and Bax significantly increased, while Nrf2, HO-1, NQO1 and Bcl-2 were significantly reduced after epilepsy. These effects were reversed by Genistein treatment. Moreover, Genistein was found to prolong seizure latency and reduce seizure intensity score and duration of generalized tonic-clonic seizures(GTCs).

Conclusions: Genistein can activate the Keap1/Nrf2 antioxidant stress pathway and attenuate the activation of microglia and astrocytes. Genistein also inhibits the JAK2-STAT3 inflammation pathway and expression of apoptotic proteins, and increases the number of surviving neurons, thus having a protective effect on epilepsy-induced brain damage.

Background

Epilepsy is a chronic neurological disease that impacts all demographics and places a heavy socioeconomic burden on both patients and the healthcare system. It is estimated that 10.5 million children worldwide suffer from epilepsy, and 80% of epilepsy patients live in low- and middle-income countries ^[1]. The causes of epilepsy are complex and diverse, including genetic and metabolic factors, immune abnormalities, trauma, infection, and systemic diseases ^[2, 3, 4]. Antiepileptic drugs can inhibit epilepsy or improve symptoms without affecting the pathological process of the disease, and about 30%

of patients have refractory epilepsy [5]. It is therefore of great importance to explore the pathogenesis of epilepsy and find new, targeted anti-epileptic drugs.

The JAK-STAT signal pathway is a cytokine-stimulated signal transduction pathway discovered in recent years. It participates in many important biological processes such as cell proliferation, differentiation, inflammation, apoptosis and immune regulation [6, 7]. Janus kinase (JAK) can sense extracellular signals by binding to receptors such as interferon, interleukin, growth factor, etc. [8], and transmit information to signal transducer and activator of transcription (STAT) [9]. Phosphorylated STATs can dimerize and translocate into nucleus where they bind to the promoter DNA sequence, causing changes in DNA transcription and activity levels, which in turn affects basic cell functions such as cell growth, differentiation and death [10]. JAK2 and STAT3 are mainly expressed in brain tissue. Studies have found that the activated JAK2/STAT3 pathway after brain injury promotes the activation and proliferation of microglia, and participates in various pathological processes such as inflammation and apoptosis in central system diseases [11, 12]. The current research on JAK2/STAT3 signaling pathway is mainly focused on hypoxic-ischemic encephalopathy, hematological tumors, immune dysfunction and cardiovascular diseases [13, 14], with few reports on developmental epilepsy.

The oxidative stress pathway plays an important role in the occurrence and development of many diseases [15, 16]. Kelch-like epichlorohydrin-related protein-1 (Keap1) is a multi-regional repressor protein of the Kelch family and a cytoplasmic inhibitor of Nrf2 [17]. Nuclear factor E2-related factor 2 (Nrf2) is a key factor in the oxidative stress response which is regulated by Keap1 and regulates the expression of antioxidant protein and phase II detoxification enzymes by interacting with the antioxidant response element ARE [18]. Under the stimulation of hypoxia, shock, infection, cytokines, etc., the Nrf2 signaling pathway can be activated to initiate the expression of multiple downstream target proteins such as hemoxygenase 1 (HO-1) and dependent reduced coenzyme/Quinone oxidoreductase 1 (NQO1) [19]. HO-1 and NQO1 can reduce the toxic effects of oxygen free radicals and endogenous toxins on neurons, thus having anti-oxidation, anti-apoptosis and anti-infection properties [20]. The current research on the Keap1/Nrf2 signaling pathway mainly focuses on cerebral hemorrhage, cerebral infarction and neurodegenerative diseases [21, 22], with again, few reports on the pathogenesis of epilepsy.

Genistein (Gen) is an isoflavone from soy extract. It is a protein tyrosinase inhibitor with strong effects of anti-angiogenesis, anti-free radical, anti-oxidation, anti-inflammatory factor release and anti-tumor [23, 24]. Studies have shown that Genistein can inhibit the excessive proliferation and activation of astrocytes and microglia induced by cerebral ischemic damage [25, 26], thereby exerting protective effects on nerve function. Genistein can also affect the expression of transcription factors [27], thereby regulating the inflammatory and immune response. However, the mechanism of Genistein in the pathogenesis of epilepsy is still unclear. The purpose of this study is to establish a developing rat epilepsy model in order to observe the regulation of the JAK2/STAT3 and Keap1/Nrf2 signaling pathways, as well as the protective mechanism of Genistein in treating epilepsy-induced brain injury.

Methods

1.1 Animals

80 healthy male Sprague–Dawley rats (20 days old, weighing 60-80g) were provided by the Experimental Animal Center, University of South China. All rats were randomly divided into 4 groups, and each group consisted of 20 animals: (1) Control group; (2) PTZ group; (3) PTZ+5mg/kg Gen group (4) PTZ +10mg/kg Gen group. The feeding conditions of all experimental animals were alternated between day and night for 12 hours. The ambient temperature was 22°C-26°C, and the humidity was approximately 55%. Rats were allowed free access to food and water. This experiment was approved by the Ethics Committee of the Second Hospital, University of South China and meets the requirements of the National Health Center.

1.2 Reagents

Pentylentetrazole and Genistein were purchased from Sigma-Aldrich (St. Louis, MO, USA). BCA Protein Quantitative Kit was purchased from Shanghai Yise Medical Technology Company (Shanghai, China). PVDF membrane and ECL chemiluminescence were purchased from Millipore (Bedford, MA). p-JAK2, p-STAT3, TNF- α , IL-1 β , Keap1, Nrf2, HO-1, NQO1, Bcl-2, caspase3, Bax and β -actin primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). NeuN (Neuronal Nuclei), GFAP (Glial Fibrillary Acidic Protein) and Iba-1 Immunofluorescence antibodies were purchased from Santa Cruz Biotechnology (CA, USA). cDNA Reverse Transcription Kit and qRT-PCR Kit were purchased from Thermo Fisher Scientific (UK). Trizol kit was purchased from Invitrogen (Grand Island, NY, USA).

1.3 Establish rat epilepsy model

Intraperitoneal injection of 35 mg/kg PTZ for 5 days to induce a developmental epilepsy model [28]. The PTZ+Gen groups were given intraperitoneal injection of Genistein (5 or 10mg/kg) 30 min before each PTZ administration. The Control group was only injected with saline. The latency, duration and intensity of each seizure were recorded, and the average values were calculated for statistical analysis. Seizure intensity scores were graded on the Racine scale [73]: Grade Ⅳ: generalized tonic-clonic seizures and falls; Grade Ⅲ: standing with forelimb clonus; Grade Ⅱ: forelimb clonus; Grade Ⅰ: rhythmic nodding; Grade 0: tremor, facial muscle twitching, chewing; Grade 0: no seizures. A Ⅳ-Ⅰgrade indicates successful seizure modeling. A total of 60 rats were injected with KA. There were 3 rats that died due to status epilepticus and 2 rats that did not exhibit seizures. The success rate of modeling was 91.6%. Finally, 16 rats in each group were selected for subsequent experiments. The rats were sacrificed with an anesthetic (1% barbital sodium, 500 mg/kg) 24h after epilepsy. The rats were then perfused intracardially with saline and hippocampal tissue were immediately collected. The samples were stored at -80°C for subsequent experiments.

1.4 Immunofluorescence staining

The rats were anesthetized with isoflurane and transcardially perfused with normal saline followed by 4% paraformaldehyde until the liver turned white. The brain tissues were removed and fixed in 4% paraformaldehyde for 24 hours, and then soaked in 30% sucrose solution for dehydration. After OCT embedding, the brain tissues were cut into 5µm thick slices on a cryostat. The tissue sections were permeabilized with 0.5% TritonX-100 at room temperature for 20 min. After blocking with 10% goat serum at room temperature for 1 h to eliminate non-specific staining, the tissue sections were incubated with primary antibodies: NeuN (rabbit anti-rat 1:500), Iba-1 (rabbit anti-rat 1:250) and GFAP (rabbit anti-rat 1:500) at 4°C overnight. After washing 3 times with PBS, the tissue sections were incubated with fluorescently labeled secondary antibody (1:1000) at 37°C for 2 h. After washing 3 times with PBS, the tissue sections were incubated with DAPI (1:1000) for 30 min to stain the nucleus. Finally, sections were mounted onto gelatin-coated glass slides and air-dried. The number of neurons, activated microglia and astrocytes were observed with a fluorescence microscope. Five high-power fields (×400) with the same area were randomly selected for each slice, and the number of NeuN, Iba1 and GFAP stained positive cells in each field were visually counted, and the average value was used for statistical analysis.

1.5 Western blot to detect the protein levels

The rats were decapitated and hippocampal tissue was separated. After adding RIPA lysate and PMSF protease inhibitor, tissue was homogenized with a glass rod until fully broken. The dissolved tissue was centrifuged at 12000 rpm for 10 min at 4°C, and the supernatant was the total protein. The supernatant was collected and the protein concentration was determined by BCA method. Stacking and separation gel were prepared according to the instructions and placed in the electrophoresis tank. After adding the protein samples and the protein marker to the sample wells, the electrophoresis tank was set to a constant voltage of 80V. Once the protein sample and the marker were electrophoresed to the separation gel position, voltage was set to 100V until the end of electrophoresis. After adding the transfer buffer to the transfer tank, the current was set to 200 mA and the protein was transferred to the PVDF membrane. The transferred PVDF membrane was put into 5% skimmed milk for blocking non-specific binding at 37°C for 2 h. The membranes were incubated at 4°C overnight with primary antibodies: p-JAK2 (1:1000), p-STAT3 (1:2000), TNF-α (1:1000), IL-1β (1:1000), Keap1 (1:1000), Nrf2 (1:1000), HO-1 (1:1000), NQO1 (1:1000), caspase3 (1:1000), Bax (1:1000), Bcl-2 (1:1000) and β-actin (1:1000). After washing 3 times with PBST, the membranes were incubated with secondary antibody (1:2000) at 37°C for 2 h. ECL luminescent solutions A and B were mixed in equal volumes, and the PVDF membrane was immersed in the luminescent solution for 5 min. After three PBST rinses, the protein bands were scanned with the Image Quant™ LAS 4000 imaging system, and the scan results were analyzed with ImageJ software.

1.6 Reverse transcription-quantitative polymerase chain reaction (qRT-PCR)

Hippocampus tissue was lysed with Trizol lysate and chloroform, and RNA was extracted through separation, precipitation and drying. According to the instructions of Thermo Scientific cDNA Synthesis Kit, sample RNA, reverse transcriptase, upstream and downstream primers were mixed to synthesize template cDNA. According to the requirements of the qRT-PCR kit, cDNA, upstream and downstream

primers were mixed to perform DNA amplification on the PCR machine. qRT-PCR primers are shown in Table 1. The qRT-PCR amplification was performed as follows: p-JAK2, HO-1:denaturation: 95°C, 45s; annealing: 56°C, 45s; extension: 72°C, 1 min; 30 cycles; p-STAT3,Bax,Nrf2: denaturation: 95°C, 45s; annealing: 54°C, 1 min; extension: 70°C, 1 min; 30 cycles; TNF- α :denaturation: 95°C, 45s; annealing: 58°C, 45s; extension: 72°C, 1 min; 30 cycles; caspase3,IL-1 β ,NQO1:denaturation: 95°C, 45s; annealing: 57°C, 45s; extension: 70°C, 1 min; 30 cycles; Keap1, Bcl-2: denaturation: 95°C, 1 min; annealing: 55°C, 45s; extension: 72°C, 45s; 30 cycles; GAPDH (Internal reference), denaturation: 95°C, 30s; annealing: 58°C, 30s; extension: 72°C, 30s; 28 cycles. Cooling at 4°C after DNA amplification. The specificity of PCR amplification products and the relative expression of RNA were analyzed by melting curve and amplification curve.

Table 1 Nucleotide sequences of qRT-PCR primers

Gene	Forward primer	Reverse primer
p-JAK2	5'- CTGCCGAGGGATGTGAGTG-3'	5'- CAGAACATTTGCCGGCTACAC-3'
p-STAT3	5'- AGGAGCATCCTGAAGCTGAC-3'	5'- CGGCAGGTCAATGGTATTGC-3'
TNF- α	5'- CCAACAAGGAGGAGAAGTTCC-3'	5'- CTCTGCTTGGTGGTTTGTCTAC-3'
IL-1 β	5'- TAAAGACCTCTATGCCAACACAGT-3'	5'- CTGACTTGGCAGAGGACAAAG-3'
Keap1	5'- AGCGCTACGATGTGGAAACA-3'	5'- GTCCAGGAACGTGTGACCAT-3'
Nrf2	5'- GACCTAAAGCACAGCCAACACAT-3'	5'- CTCAATCGGCTTGAATGTTTGTC-3'
HO-1	5'- TGTCCCAGGATTTGTCCGAG-3'	5'- ACTGGGTCTGCTTGTTCGCT-3'
NQO1	5'- GGGGACATGAACGTCATTCTCT-3'	5'- AGTGGTGACTCCTCCCAGACAG-3'
Bcl-2	5'- TCGCCCTGTGGATGACTGA-3'	5'- CAGAGACAGCCAGGAGAAATCA-3'
caspase3	5'- TGTCGATGCAGCAAACCTCA-3'	
Bax	5'- TCCACCAAGAAGCTGAGCGAG-3'	5'- GTCCAGCCCATGATGGTTCT-3'
GAPDH	5'- AGAAGGCTGGGGCTCATTG-3'	5'- AGGGGCCATCCACAGTCTTC-3'

1.7 Statistical analysis

All the data analyses were performed using SPSS 20.0 statistical software. The data were expressed as Mean \pm SD. Means comparison between two groups were analyzed by t-test and P<0.05 was considered statistically significant. Means comparison between multiple groups were analyzed by one-way analysis of variance (ANOVA), and the Bonferroni post-test correction was used to reduce the likelihood of false positives. There were 5 comparisons for Western blot, qRT-PCR, TUNEL and Immunofluorescence staining, and the significance level was adjusted accordingly to P<0.01.

Results

2.1 Genistein inhibits seizure activity

In this experiment, PTZ was used to induce epilepsy and Genistein was used for pretreatment in developing rats. The seizure latency, grade scores and duration of the first generalized tonic-clonic seizure (GTCs) were recorded, and the average value was used for statistical analysis. The results were as follows: Seizure grade scores: Control group 0; PTZ group 4.09 ± 0.43 ; PTZ+5mg/kg Gen group 3.50 ± 0.42 ; PTZ+15mg/kg Gen group 3.17 ± 0.62 ; Seizure latency: PTZ group 253.7 ± 77.24 s; PTZ +5mg/kg Gen group 562.5 ± 179.5 s; PTZ+15mg/kg Gen group 747.6 ± 131.3 s; Duration of the first GTCs: PTZ group 28.56 ± 8.71 s; PTZ+5mg/kg Gen group 17.50 ± 9.62 s; PTZ+15mg/kg Gen group 5.69 ± 5.15 s.

Compared with the PTZ group, the Genistein treatment groups reduced seizure intensity and duration, and prolonged seizure latency (Figures 1-3). Further comparison found that the Genistein dose of 15mg/kg had a greater therapeutic effect than 5mg/kg. These results suggest that Genistein can inhibit seizure activity.

2.2 Genistein increases mature neuronal cell count and reduces activated microglia and astrocytes

Immunofluorescence staining was used to observe the number of mature neurons, activated microglia and astrocytes in the hippocampal CA1 region 24 h after epilepsy (Figure 4, 5, 6). The results are as follows: in the Control group, the number of mature neurons (NeuN positive cells) was normal and the distribution was neat and orderly (Figure 4A1), while activated microglia (Iba-1 positive cells) and astrocytes (GFAP positive cells) were almost invisible (Figure 5A1,6A1); In the PTZ group, the number of mature neurons was significantly reduced (Figure 4B1) and the distribution was disordered; activated microglia and astrocytes were significantly increased ($p < 0.01$), with the cell volume of microglia becoming larger with an amoeba or round appearance, and shorter, thickened or fused cell protrusions (Figure 5B1). Compared with the PTZ group, the number of neurons significantly increased in the Genistein (5 or 15mg/kg) treatment groups (Figure 4C1,4D1) ($p < 0.01$); activated microglia were significantly reduced in the PTZ+15mg/kg Gen group (Figure 5D1) ($p < 0.01$) and the cell volume became smaller and the protrusions more slender; activated astrocyte were also significantly reduced in the Genistein (5 or 15mg/kg) treatment groups (Figure 6C1,6D1) ($p < 0.05$). Further comparison found that 15mg/kg Genistein was more effective than the 5mg/kg dose.

Immunofluorescence staining suggests that Genistein can increase mature neuronal cells and reduce activated microglia and astrocytes, thus supporting its protective effect on brain damage post epilepsy.

2.4 Genistein inhibits the expression of JAK2/STAT3 signaling pathway

To explore the effect of Genistein on the JAK2/STAT3 signaling pathway, Western blot and qRT-PCR were used to determine the protein and mRNA levels of p-JAK2, p-STAT3, TNF- α and IL-1 β in the hippocampus after PTZ-induced epilepsy (Figures 7 and 8). The optical density of the band was used to further analyze the relative expression of the protein (Figure 7B). Our findings indicate that the protein and mRNA levels

of p-JAK2, p-STAT3, TNF- α and IL-1 β increased significantly in the PTZ group and these increases were attenuated by Genistein treatment (Fig. 7B and 8, $p < 0.01$). Further comparison revealed that the protein levels of p-JAK2 and IL-1 β , and the mRNA levels of p-JAK2, TNF- α and IL-1 β in the PTZ+15mg/kg Gen group were significantly lower than that in the PTZ+5mg/kg Gen group ($p < 0.01$).

Western blot and qRT-PCR results thus indicate that Genistein can inhibit the expression of the JAK2/STAT3 signaling pathway on PTZ-induced epilepsy in developing rats.

2.5 Genistein activates the Keap1/Nrf2 signaling pathways

To further investigate the effect of Genistein on the Keap1/Nrf2 antioxidant stress pathway, Western blot and qRT-PCR were used to detect the protein and mRNA levels of Keap1, Nrf2, HO-1 and NQO1 in the hippocampus after epilepsy (Figures 9 and 10). The results were as follows: \square The protein and mRNA levels of Keap1 increased significantly in the PTZ group compared to the Control group ($p < 0.01$), and this increase was inhibited by Genistein treatment ($p < 0.01$) (Fig. 9B and 10); \square The protein and mRNA levels of Nrf2, HO-1 and NQO1 in the PTZ group were significantly lower than the Control group levels ($p < 0.01$), and these expression changes were reversed by Genistein treatment; (Fig. 9B and 10); \square Further comparison revealed that the protein and mRNA levels of Keap1 and NQO1 were significantly different in PTZ+5mg/kg Gen group and PTZ+15mg/kg Gen group ($p < 0.01$); the protein level of HO-1 and the mRNA level of Nrf2 in the PTZ+15mg/kg Gen group was significantly higher than that in the PTZ+5mg/kg Gen group ($p < 0.01$).

These result suggests that Genistein can activate the Keap1/Nrf2 antioxidant stress pathway after PTZ-induced epilepsy. Furthermore the inhibitory effect is dose-dependent, with 15mg/kg Genistein having a greater inhibitory effect.

2.6 Genistein inhibits the expression of apoptotic markers

In order to explore the effects of Genistein on apoptosis-related proteins, Western blot and qPCR were used to measure the protein and mRNA levels, respectively, of caspase3, Bax and Bcl2 in the hippocampus after epilepsy (Figure 11, 12). The results were as follows: \square The protein and mRNA levels of caspase3 and Bax in the PTZ group increased significantly after epilepsy ($p < 0.01$), while Genistein treatment was shown to reduce the expression of both; \square The protein and mRNA levels of Bcl2 in the PTZ group decreased significantly compared to the Control group ($p < 0.01$), and these expression changes were reversed by Genistein treatment (Fig. 11 and 12); \square Further comparison found that Bax protein and caspase3 mRNA were significantly lower, and Bcl2 mRNA expression was significantly higher in the PTZ+15mg/kg Gen group than in the PTZ+5mg/kg Gen group.

These results demonstrate that Genistein can reduce the ratio of apoptotic proteins caspase3 and Bax to anti-apoptotic protein Bcl2, thereby exerting a protective effect on cell apoptosis. At the same time, a heightened treatment effect is correlated with a heightened Genistein dose.

Discussion

Epilepsy is a chronic brain disease caused by the abnormal discharge of brain neurons. At present, the pathogenesis of epilepsy is not clear. The existing antiepileptic drugs primarily suppress neuronal excitability by regulating neurotransmitter receptors (such as glutamate, GABA and acetylcholine) or ion channels [29, 30, 31]. These are in fact anticonvulsant medications rather than antiepileptic treatments, as they do not target the implicit underlying etiology. Epilepsy can cause the activation and proliferation of microglia and astrocytes, which are involved in various pathological processes such as inflammation and apoptosis in central system diseases [32, 33]. At the same time, epileptic seizures will increase oxidative stress and promote the production of reactive oxygen species, leading to excessive neuronal excitement, oxidative damage, cells apoptosis and long-term intracranial biochemical changes [34, 35]. Therefore, it is believed that the inflammatory and oxidative stress responses are the underlying mechanisms of epilepsy neuropathology. In this experiment, we chose the PTZ-induced epilepsy model as it is similar to the human epilepsy model [36], and PTZ is related to nerve excitotoxicity and the expression of reactive oxygen species [37].

JAK2/STAT3 is an important inflammatory response-related pathway. The activation of this pathway can initiate gene transcription in the nucleus, and regulate the inflammatory response and the expression of apoptotic factors [38]. JAK2 is a kind of non-receptor tyrosine protein kinase family. STAT3 is a denucleotide binding protein, which is the substrate and downstream factor of JAK2 [39]. JAK2 and STAT3 are widely distributed throughout the central nervous system, and can be activated by many cytokines and growth factors (such as interleukins, colony stimulating factors, growth hormones, etc.) [40]. Activated JAK2 can activate STAT3, and transmit extra-membrane stimulus signals into cells through the tyrosine residues of various target proteins, leading to the phosphorylation of JAK2 and STAT3. p-STAT3 dimerizes and transfers to the nucleus, which can bind specific promoter sequence and regulate gene transcription [41]. In this experiment, the results of Western blot and qRT-PCR showed that the expression of the JAK2/STAT3 signaling pathway as well as apoptotic proteins caspase3 and Bax, increased significantly after epilepsy. This implicates the JAK2/STAT3 signaling pathway in the pathological process of epilepsy. In combination with experimental results, we considered the following possible mechanisms: ¶Epilepsy can induce the activation of JAK2/STAT3 signaling. The activated JAK2/STAT3 signal promotes the expression of downstream inflammatory factors TNF- α and IL-1 β [42]. At the same time, the high expression of TNF- α and IL-1 β can bind to their receptors, and further activate JAK2 and STAT3, leading to their phosphorylation [43]. The combination of p-JAK2 and p-STAT3 with DNA increases the expression of cytokine genes and produces more interleukins and cytokines. This vicious cycle can lead to persistent inflammation that is difficult to control. ¶The downstream signals regulated by JAK2/STAT3 also include the apoptotic targets caspase3, Bax, and Bcl-2 family proteins. Therefore, the activation of JAK2/STAT3 can lead to neuronal apoptosis and aggravate the pathological process of brain injury [44]. ¶JAK2/STAT3 signaling can mediate the activation of microglia and astrocytes, which can in turn, secrete TNF- α and IL-1 β , leading to neuroinflammation and brain damage after epilepsy. Yang's research shows that JAK2/STAT3 signaling can cause brain damage and behavioral

abnormalities through increasing the expression of IL-1 β in neonatal rats with hypoxic-ischemic encephalopathy [45] while Liu's research shows that inhibiting the JAK2/STAT3 signaling pathway can reduce seizure-induced brain injury [46]. These research results are consistent with our findings and support our proposed mechanisms of epileptic-pathology.

Keap1 is a multi-domain repressor protein of the Kelch family, which binds to Nrf2 through the Cul3 ubiquitin ligase containing E3. Nrf2 is an important transcription factor that regulates cellular anti-oxidative stress and can promote the expression of a series of antioxidant protective proteins [47]. At the same time, it can coordinate many protective detoxification and anti-inflammatory genes, and synergistically improve the efficiency of the cellular defense system [48]. Under physiological conditions, Keap1 combines with and inhibits Nrf2. Under the influence of external oxidative stressors, Nrf2 is decoupled with Keap1 and combined with the antioxidant response element ARE to activate the Nrf2 signaling pathway, thereby increasing the expression of antioxidant proteins HO-1, SOD, NQO1, etc., and reducing oxidative damage and accumulation of toxicity metabolites [49,50]. In a study by Wang et al. when the amygdala was rapidly ignited, the protein and gene levels of Nrf2, HO-1 and NQO1 increased significantly in the hippocampus, which confirmed that the Nrf2 signaling pathway plays an important role in the early stages of epileptic seizures [51]. Wu et al. found that activating the Nrf2-ARE signaling pathway had anticonvulsant effects and improved the cognitive function of epileptic rats, while Nrf2 knockout mice had more severe seizures and obvious cognitive impairment [52]. In our experiment, the results of Western blot and qPCR showed that the expression of Nrf2, HO-1, NQO1 decreased and Keap1 increased significantly after epilepsy, suggesting inhibition of the Nrf2 antioxidant pathway, and activation of oxidative stress pathways, which is consistent with previous studies. At the same time, the Keap1/Nrf2 signaling pathway was activated after Genistein treatment, and the expression of IL-6 inflammatory mediators was reduced. This indicates that the overexpression of Nrf2 can inhibit the inflammatory response against post-epileptic brain injury, and this effect may be related to the inhibition of JAK3/STAT2 and TLR4/NF-kB inflammation signal pathway [53].

Microglia are the key regulators of immune response in the central nervous system. Under normal circumstances, microglia are in a static state but can be activated when subjected to abnormal stimulation, leading to rapid proliferation and migration to the injured site, and mediating neuroinflammation and neuronal apoptosis [54]. Therefore, inhibiting the activation and proliferation of microglia may be an effective measure to reduce epilepsy-induced brain injury. Astrocytes are macroglial cells in the central nervous system. They play an important role in maintaining the stability of the internal environment, forming the blood-brain barrier, and transmitting nerve excitation [55]. Astrocytes are the place where glutamate (Glu) and γ -aminobutyric acid (GABA) are metabolized. They have a spatial buffering effect on extracellular potassium ions and can maintain ion balance around neurons [56, 57]. Astrocytes can participate in the immune response of the central nervous system, and can cause neuroinflammation under the stimulation of central nervous system damage, infection, toxins or autoimmunity [58]. In the pathological process of epilepsy, K⁺ channels are involved in regulating cell membrane resting potentials and the repolarization process of action potentials, and are closely related

to the excitability of neurons [59]. At the same time, during epileptic seizures, the excitatory and inhibitory amino acids are unbalanced within and outside the cell, and as mentioned, the inflammatory pathway is activated [60]. Thus we propose that astrocytes are closely related to the pathological process of epilepsy. In this experiment, the immunofluorescence results showed that Iba-1 and GFAP positive cells increased significantly after epilepsy, which confirmed that the activation and proliferation of microglia and astrocytes were involved in the pathological process of PTZ induced-epilepsy. Activated microglia and astrocytes can cause neuronal damage and apoptosis through pro-inflammatory mediators, cytokines and ROS [61]. We found that NeuN positive cells decreased significantly after epilepsy, indicating that the number of surviving neurons decreased. Meanwhile, the results of Western blot and qPCR showed that the apoptotic proteins caspase3 and Bax, and the inflammatory factor, IL-6 increased significantly, confirming that neuronal apoptosis and inflammation are involved in the pathological process of epilepsy. The specific mechanism considers that activated astrocytes and microglia secrete a variety of immune effector molecules, such as interleukins IL-1, IL-6, IL-8, NF-kB and oxygen free radicals [62], which can damage neurons, glial cells and the blood-brain barrier, leading to local or extensive damage of the central nervous system. Furthermore, abnormal glial cell function can cause a decrease in potassium buffer capacity or an excessive intake of GABA, which may induce seizures and aggravate brain damage [63]. At the same time, the production of inflammatory factors can further activate microglia and astrocytes and promote the pathological process of epilepsy [64]. These potential mechanisms of action are consistent with the results of our study.

Genistein is a natural isoflavone compound that has anti-tumor, -oxidation, and -inflammatory properties, and offers synaptic protection, a reduction of astrocyte aggregation and improvements in learning and memory [65]. Some studies show that Genistein can reduce the NF-kB-induced inflammatory response by reducing ROS levels, blocking mitochondrial-dependent apoptosis and thereby reducing the area of cerebral infarction [66]. Genistein can also directly act on vascular endothelial cells, alleviating oxidative stress, inflammation and vascular damage by increasing the protein expression of eNOS [67]. At present, the research on Genistein mainly focuses on ischemic stroke, brain injury and Alzheimer's disease [68, 69], with few reports on epilepsy. In this experiment, we observed that activated microglia and astrocytes, expression of JAK2/STAT3 inflammation pathway, and apoptotic caspase3 and Bax increased significantly after epilepsy, while the number of neurons, expression of Nrf2 anti-oxidative stress pathway and anti-apoptotic protein Bcl-2 were reduced. Genistein was found to reverse these pathological processes, thereby playing a protective effect in epilepsy-induced brain damage. We consider the following as potential underlying mechanisms for this effect: ☒ Genistein reduces the expression of inflammatory factors by inhibiting the JAK2/STAT3 signaling pathway; Masakatsu demonstrated that Genistein can block JAK2 phosphorylation and EPO-induced glutamate release in a cerebral ischemia model, indicating Genistein has a neuroprotective effect consistent with our findings [70]; ☒ Genistein inhibits mitochondrial damage and the initiation of apoptosis programs by increasing the synthesis of anti-apoptotic proteins, thereby increasing the number of surviving neurons [71]; ☒ Genistein increases the expression of antioxidant proteins HO-1 and NQO1 and inhibits the generation of reactive oxygen species,

thereby reducing the oxidative damage process induced by epilepsy; in Miao's research, Genistein increased the expression of Nrf2 and reduced brain damage caused by oxidation stress^[72], which is consistent with our study; Genistein reduces the expression of inflammatory factors by inhibiting the activation of astrocytes and microglia; Studies have also shown that Genistein inhibits the Ca²⁺ influx and glutamate release of hippocampal synaptosomes^[74,75], thereby reducing seizures. This experiment proved that Genistein can reduce the seizure intensity and seizure duration, and prolong seizure latency, which is consistent with results in the literature. At the same time, it was found that Genistein has a greater therapeutic effect at a dose of 15 mg/kg compared to 5 mg/kg, indicating that the protective effect of Genistein on brain damage after epilepsy may be dose-dependent.

Conclusions

In conclusion, Genistein can reduce the intensity and duration of seizures, inhibit the activation of microglia and astrocytes as well as the expression of JAK2/STAT3 inflammatory pathways, while activating the Keap1/Nrf2 oxidative stress pathway, and increasing anti-apoptotic proteins and neuronal survival. This experiment thereby reveals the potential protective effects and underlying mechanisms of Genistein on brain damage after developmental epilepsy, and also provides a theoretical basis for the development of new anti-epileptic drugs.

Abbreviations

Gen: Genistein; PTZ: Pentylentetrazole; Kelch-like epichlorohydrin-related protein-1:Keap1; Nuclear factor E2-related factor 2:Nrf2; hemeoxygenase 1:HO-1; coenzyme/Quinone oxidoreductase 1:NQO1; generalized tonic-clonic seizures: GTCs; Janus kinase: JAK; signal transducer and activator of transcription: STAT; glutamate: Glu; γ -aminobutyric acid : GABA; NeuN (Neuronal Nuclei), GFAP (Glial Fibrillary Acidic Protein)

Declarations

Ethics approval and consent to participate

All animal breeding, care, feeding, and surgical procedures were approved by the Laboratory Animal Users Committee at the University of South China.

Consent for publication

All authors agree to publish the article.

Availability of data and material

The data and material are available from the corresponding author upon reasonable request.

Competing interests

None of the authors has any conflict of interest.

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Authors' contributions

Qing-peng Hu participated in the experiment design, data collection, performed experiments and wrote the first draft of the manuscript.

Xiang-yi Huang participated in the experiment design, statistical analysis, data collation and proofing.

Wei Feng, Feng-fang Chen, Hong-xia Yan, Xin-Zhang, Zhou-yuan Yang performed experiments and gathered data.

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Figures

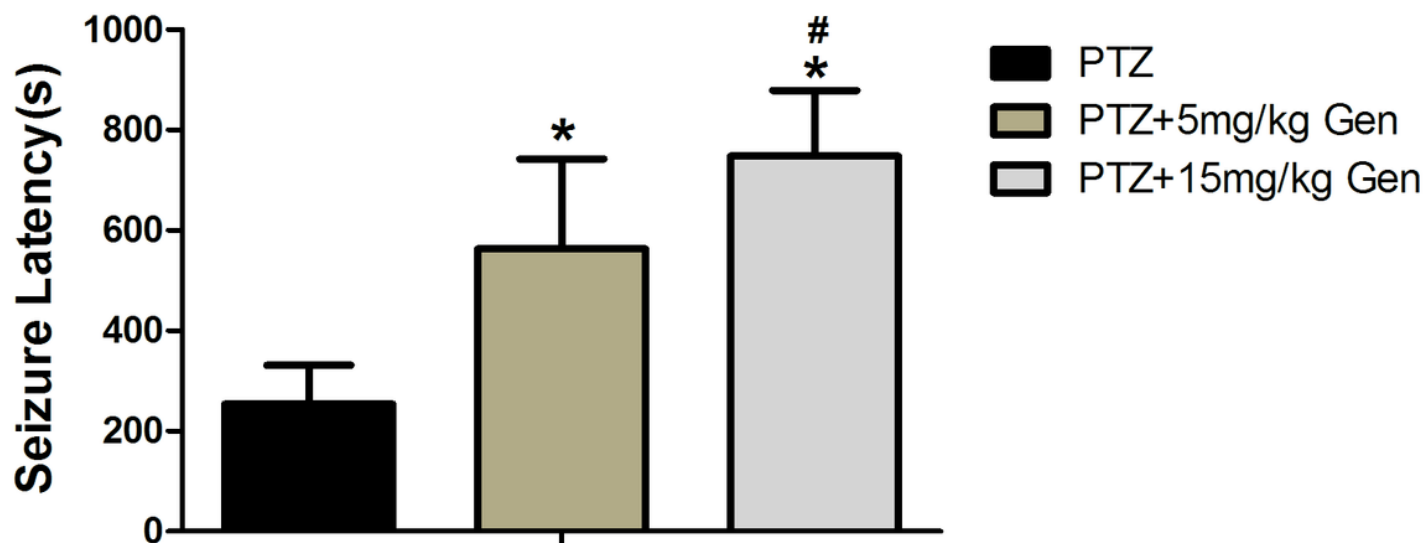


Figure 1

Genistein increases seizure latency. PTZ 35mg/kg induced epilepsy and different doses of Genistein (5, 15 mg/kg) were used for pretreatment. Seizure latency was assessed after PTZ administration. Data were expressed as mean±SD (n=16). *P<0.05, compared with PTZ group; #P<0.05, compared with PTZ+5mg/kg Gen group.

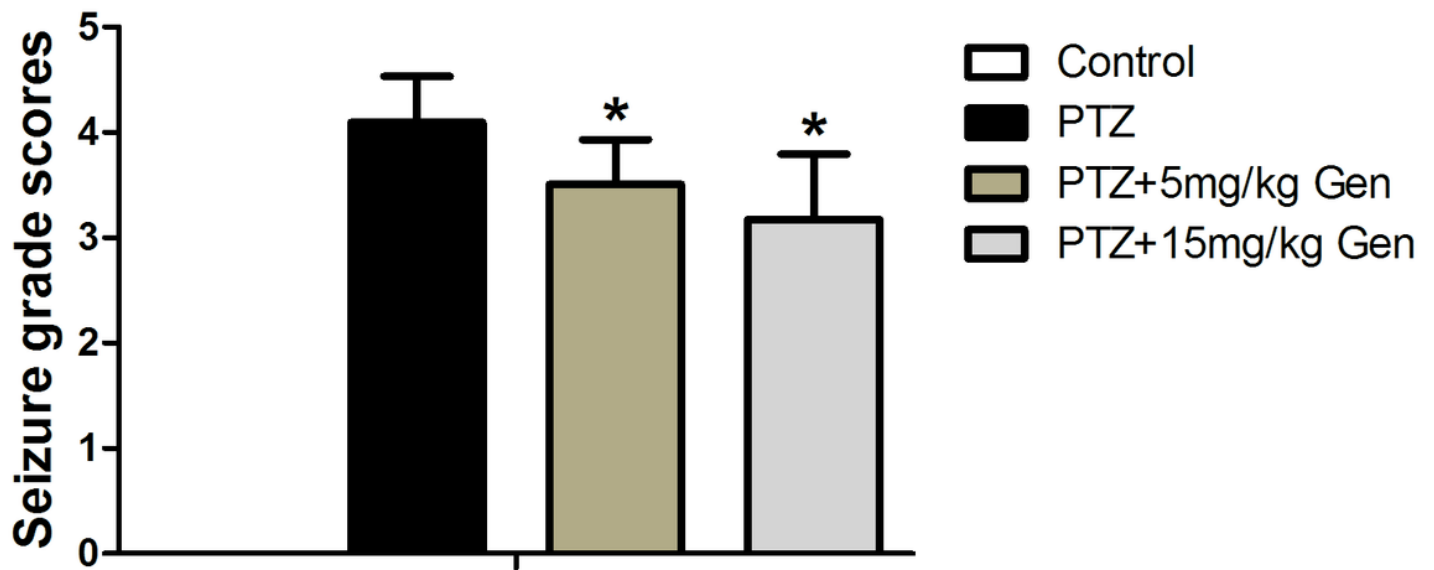


Figure 2

Genistein reduces seizure grade scores. PTZ 35mg/kg induced epilepsy and different doses of Genistein (5, 15 mg/kg) were used for pretreatment. Seizure grade scores were assessed after PTZ administration. Data were expressed as mean±SD (n=16). *P<0.05, compared with the PTZ group.

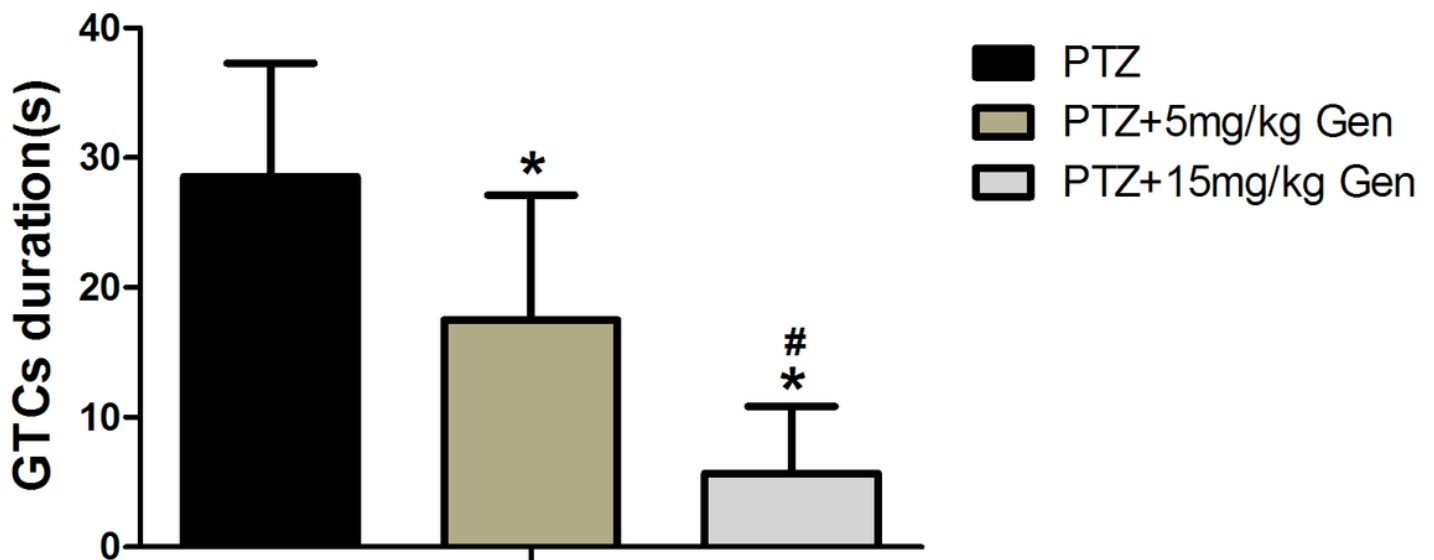


Figure 3

Genistein decreases the duration of the first GTCs. PTZ 35mg/kg induced epilepsy and different doses of Genistein (5, 15 mg/kg) were used for pretreatment. Duration of the first GTCs was assessed. Data were

expressed as mean±SD (n=16). *P<0.05, compared with PTZ group; #P<0.05, compared with PTZ+5mg/kg Gen group.

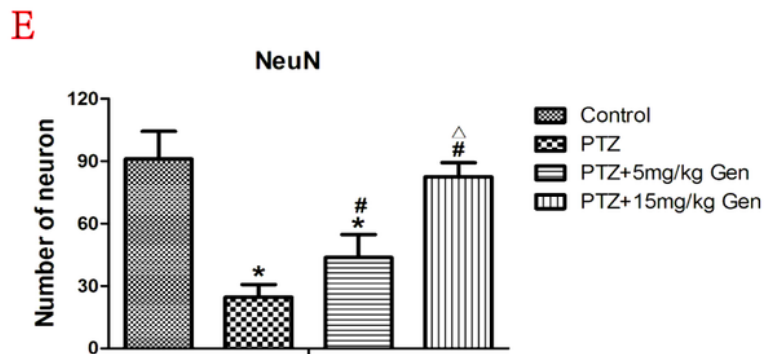
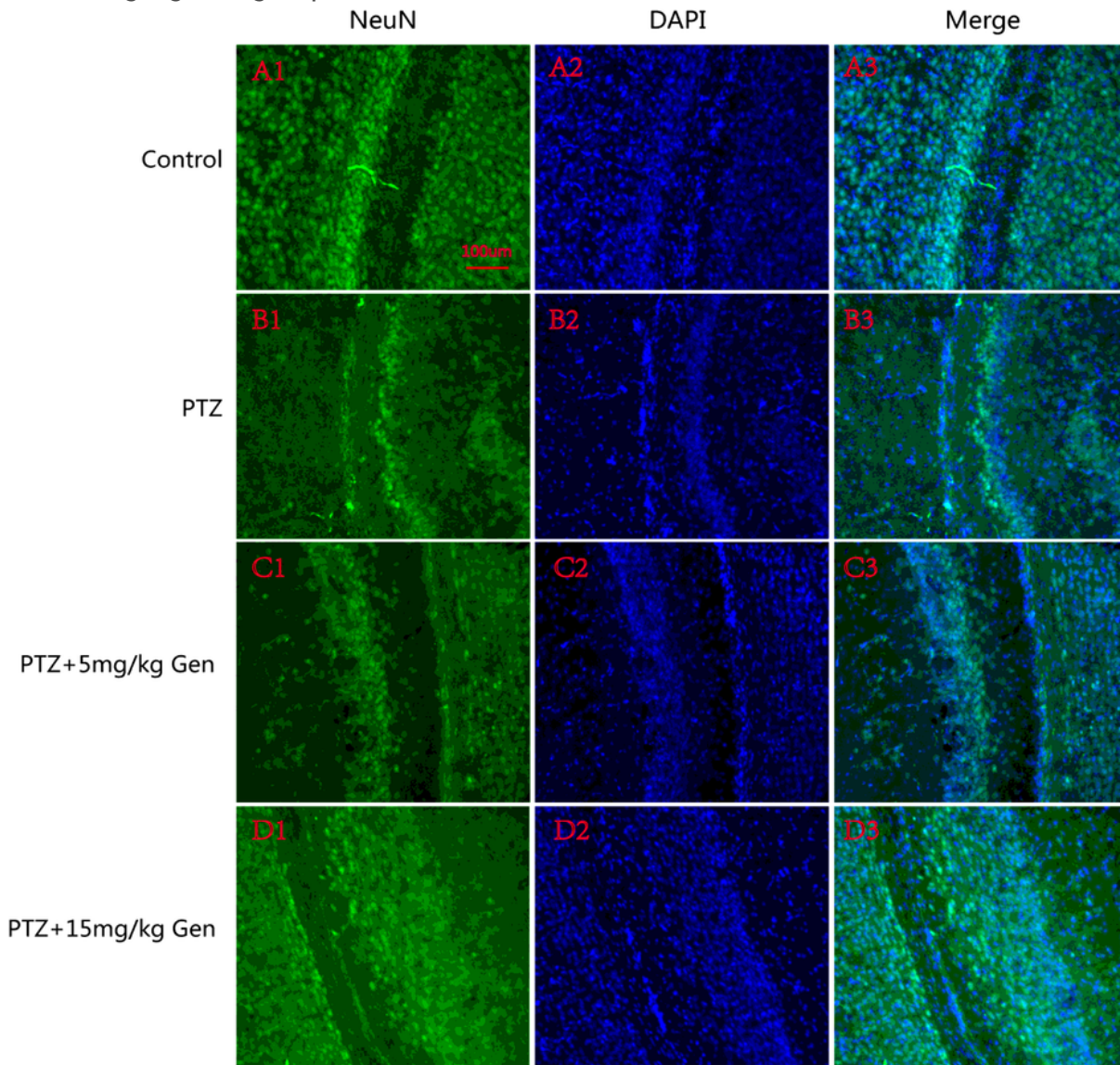


Figure 4

Genistein increases mature neuronal cells. Immunofluorescence staining was used to observe neurons in the hippocampal CA1 region. A. Control group B. PTZ group C. PTZ+5mg/kg Gen group D. PTZ+15mg/kg Gen group. F. Quantification of NeuN positive cells for each group. Data were expressed as mean±SD,

n=5. Scale bar = 100 μ m. *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15mg/kg) vs. PTZ group; Δ P<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

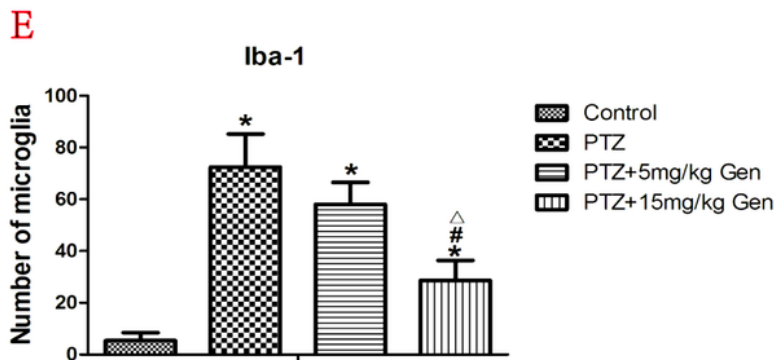
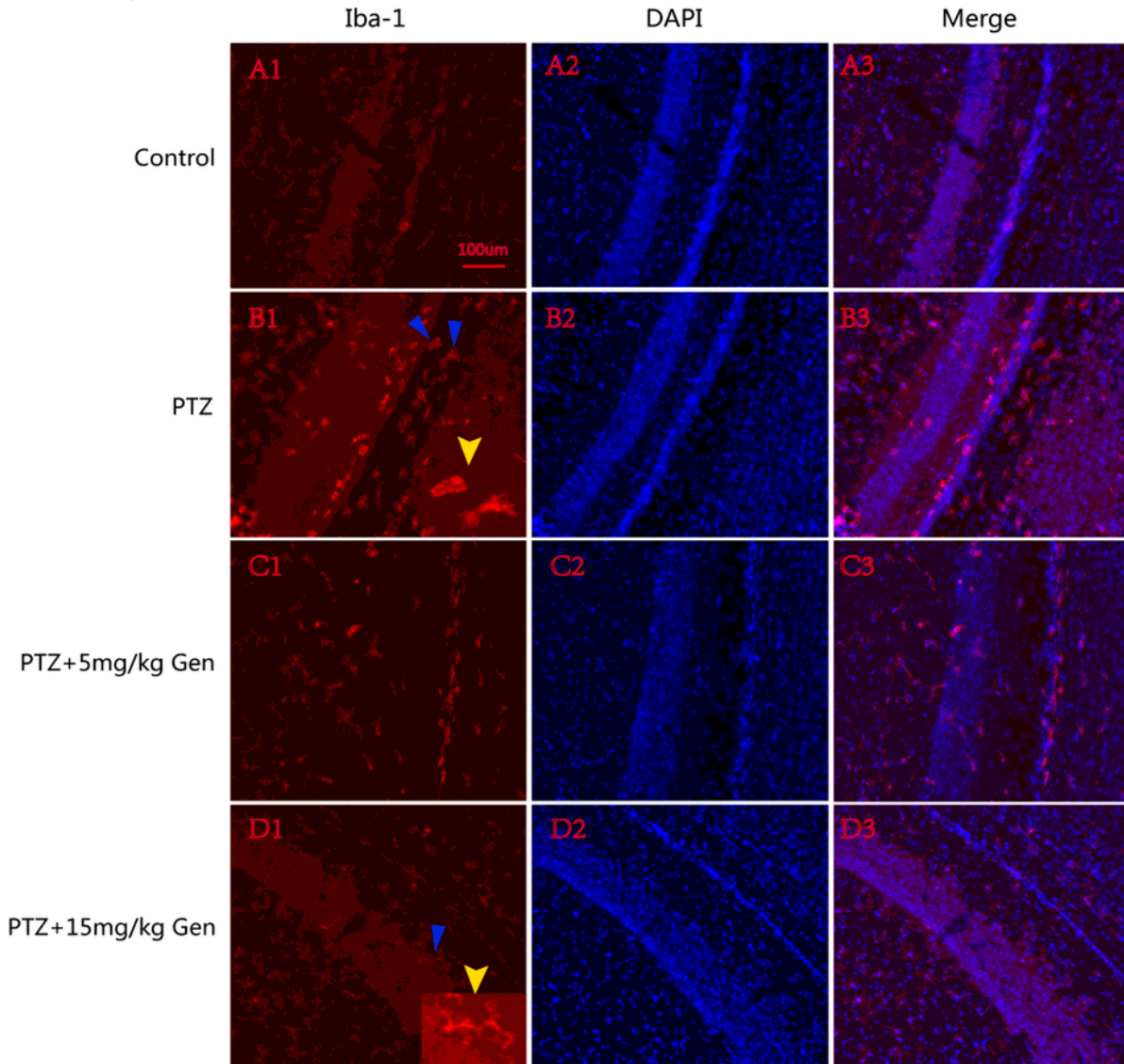


Figure 5

Genistein reduces activated microglia. A. Control group B. PTZ group C. PTZ+5mg/kg Gen group D. PTZ+15mg/kg Gen group. F. Quantification of Iba-1 positive cells for each group. Insets in B1 and D1 show morphological change after PTZ and Genistein administration under higher magnification. Data

were expressed as mean±SD, n=5. Scale bar = 100 μm. *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15mg/kg) vs. PTZ group; ΔP<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

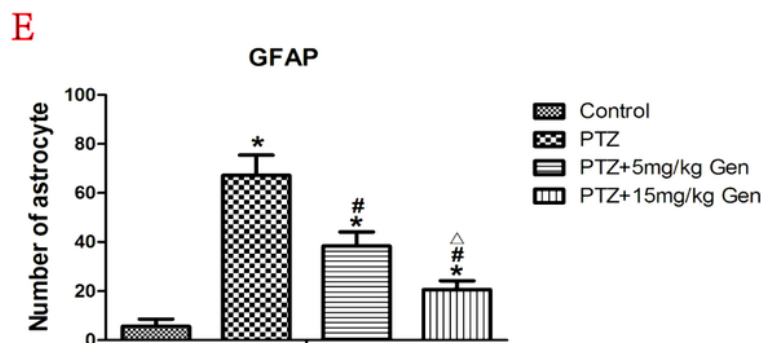
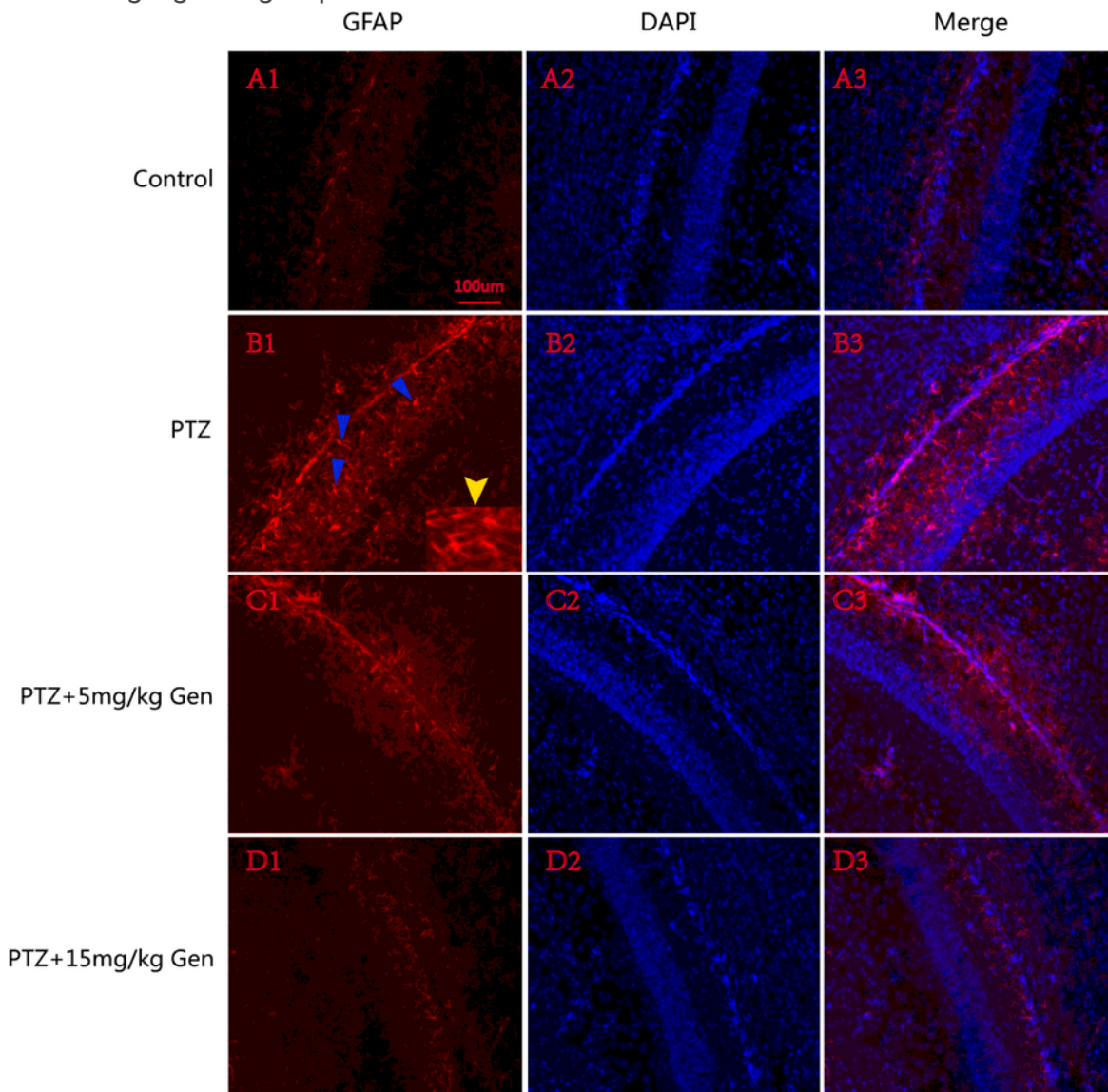


Figure 6

Genistein reduces activated astrocytes. A. Control group B. PTZ group C. PTZ+5mg/kg Gen group D. PTZ+15mg/kg Gen group. F. Quantification of GFAP positive cells for each group. Insets in B1 show

morphological change after PTZ administration under higher magnification. Data were expressed as mean±SD, n=5. Scale bar = 100 μm. *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15mg/kg) vs. PTZ group; ΔP<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

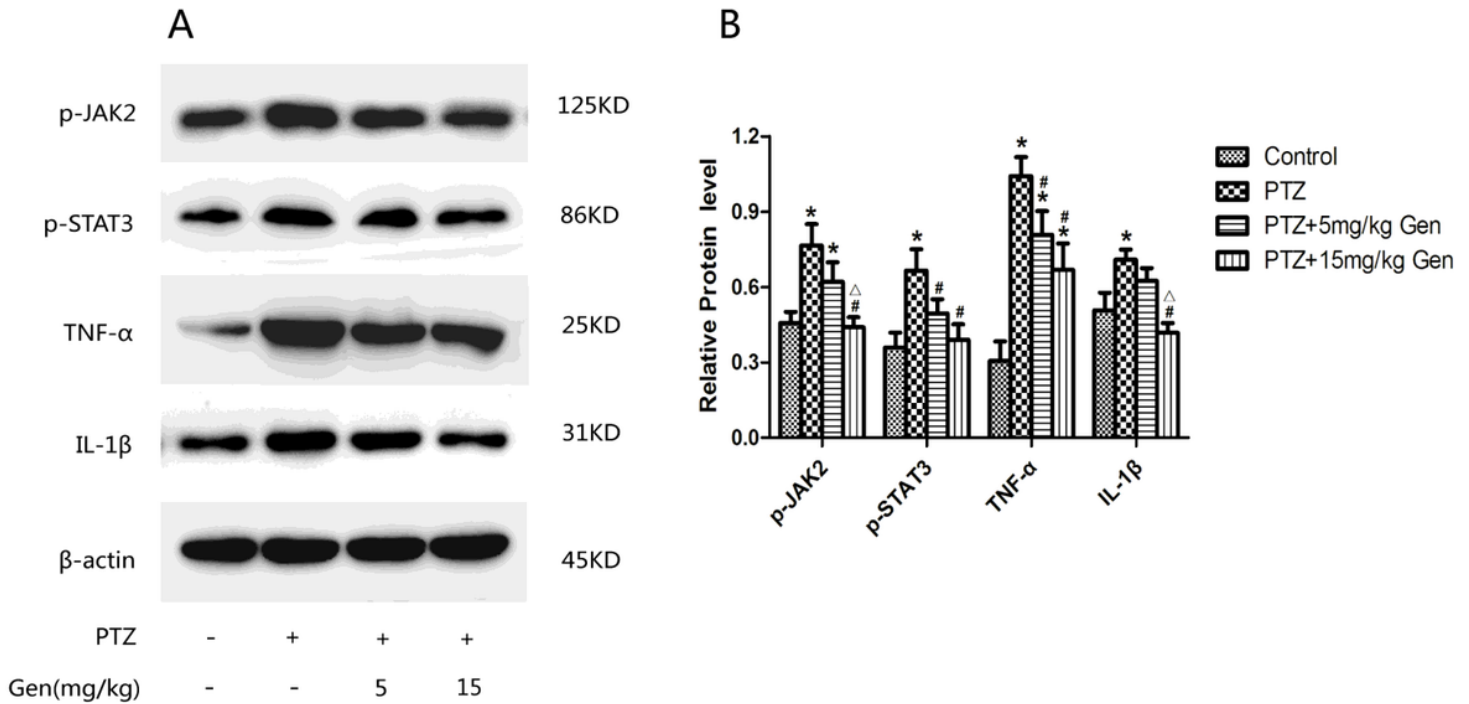


Figure 7

Genistein inhibits protein levels of p-JAK2, p-STAT3, TNF-α and IL-1β after PTZ-induced epilepsy. Protein levels of p-JAK2, p-STAT3, TNF-α and IL-1β were measured 24 h after PTZ injection. A. Protein bands following Western blot analysis. B. Relative protein levels (optical density). Measurement data were expressed as mean±SD (n=16). *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15 mg/kg) vs. PTZ group; ΔP<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

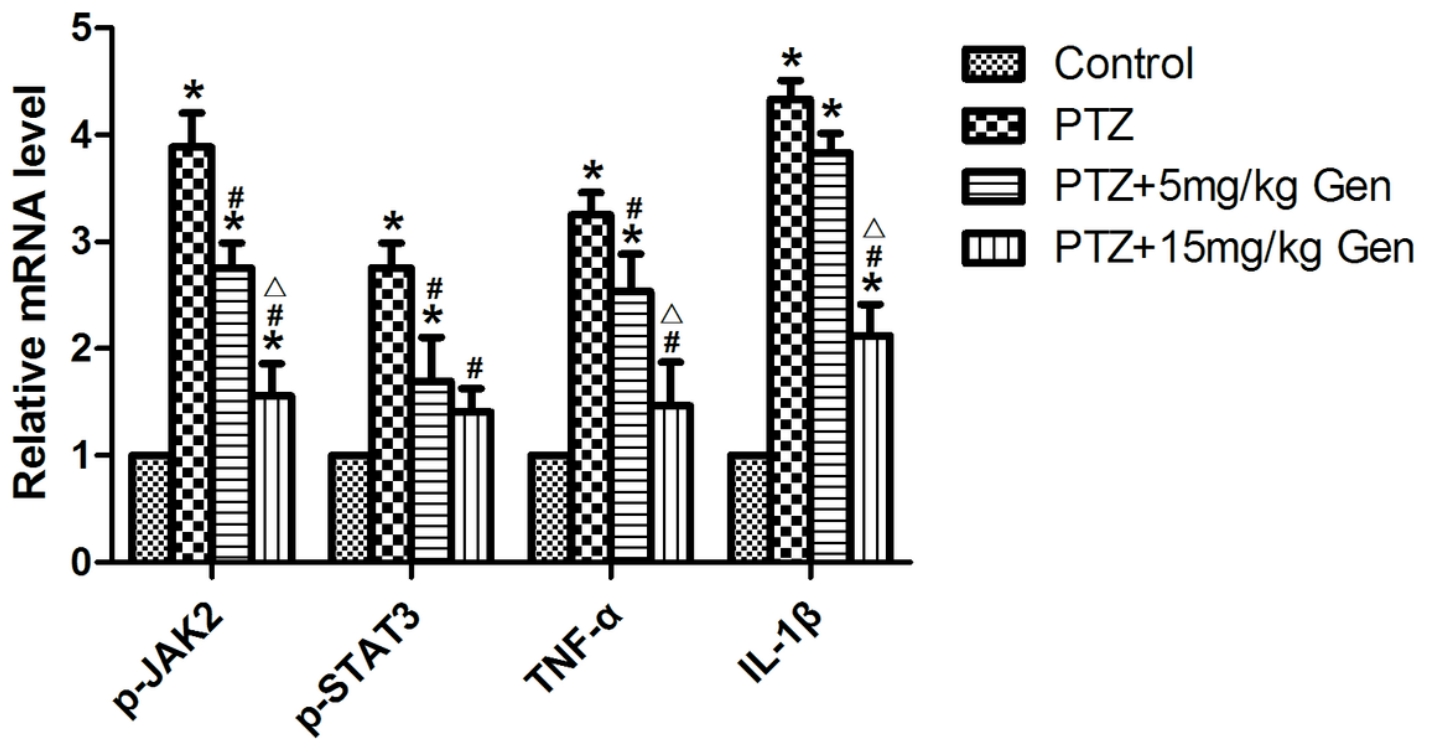


Figure 8

Genistein reduces mRNA levels of p-JAK2, p-STAT3, TNF- α and IL-1 β in the hippocampus. Measurement data were expressed as mean \pm SD (n=16). *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15 mg/kg) vs. PTZ group; Δ P<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

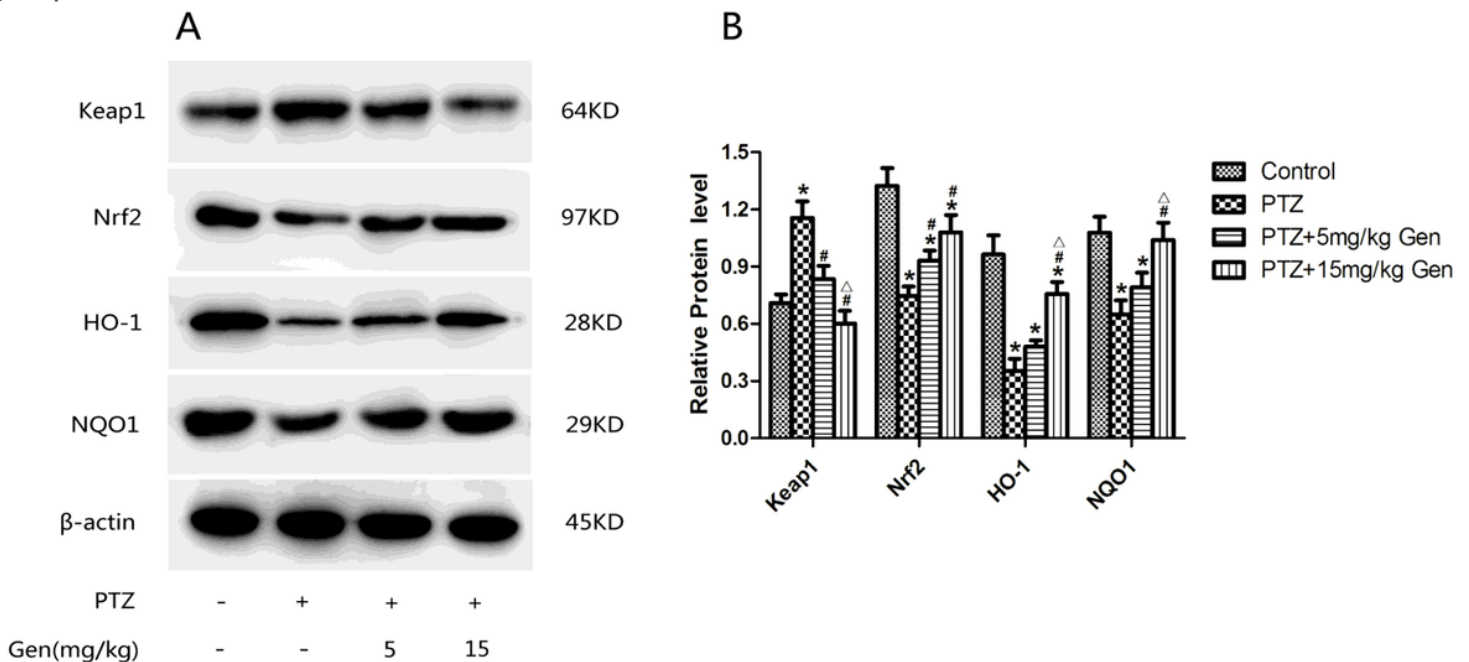


Figure 9

Genistein activates the Keap1/Nrf2 signaling pathways. Protein levels of Keap1, Nrf2, HO-1 and NQO1 were measured after PTZ-induced epilepsy. A. Protein bands following Western blot analysis. B. Relative protein levels (optical density). Measurement data were expressed as mean±SD (n=16). *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15 mg/kg) vs. PTZ group; ΔP<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

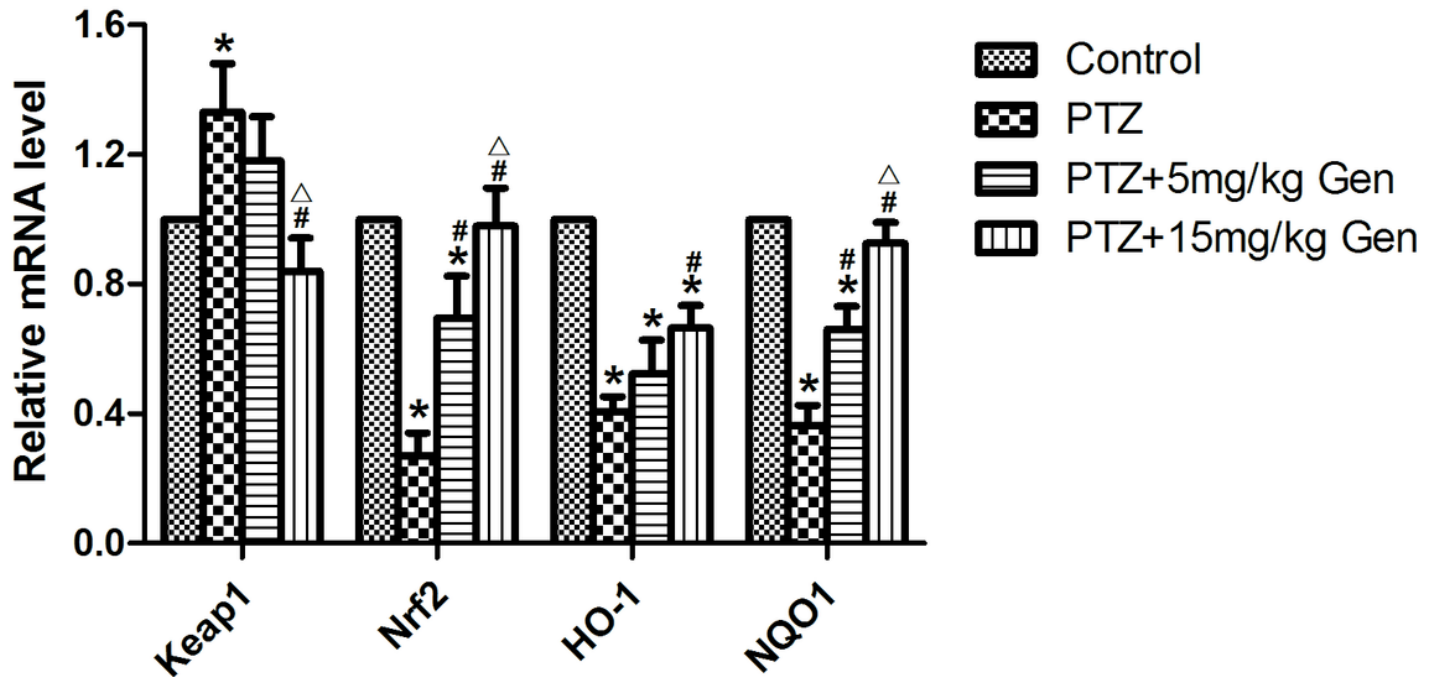


Figure 10

mRNA levels of Keap1, Nrf2, HO-1 and NQO1 were measured after PTZ-induced epilepsy. Measurement data were expressed as mean±SD (n=16). *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15 mg/kg) vs. PTZ group; ΔP<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

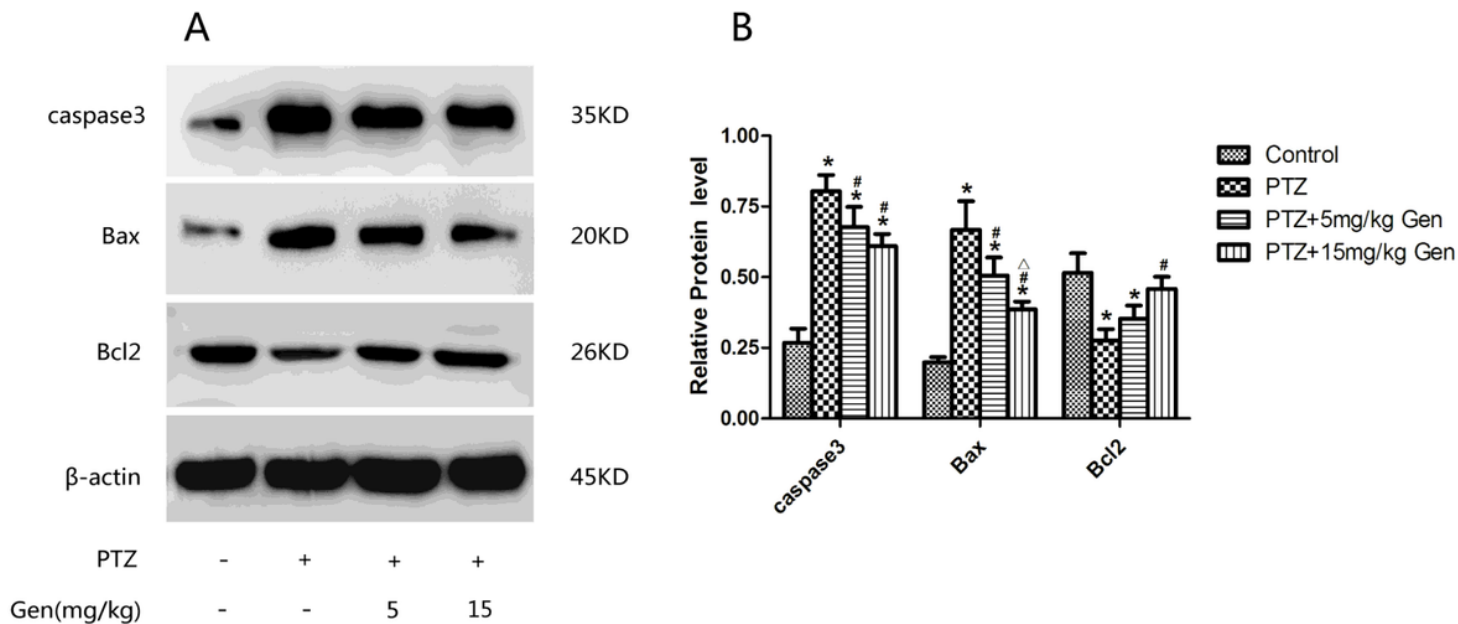


Figure 11

Genistein inhibits the expression of apoptosis proteins. Protein levels of caspase3, Bax and Bcl2 were measured after PTZ-induced epilepsy. A. Protein bands following Western blot analysis. B. Relative protein levels (optical density). Measurement data were expressed as mean \pm SD (n=16). *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15 mg/kg) vs. PTZ group; Δ P<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.

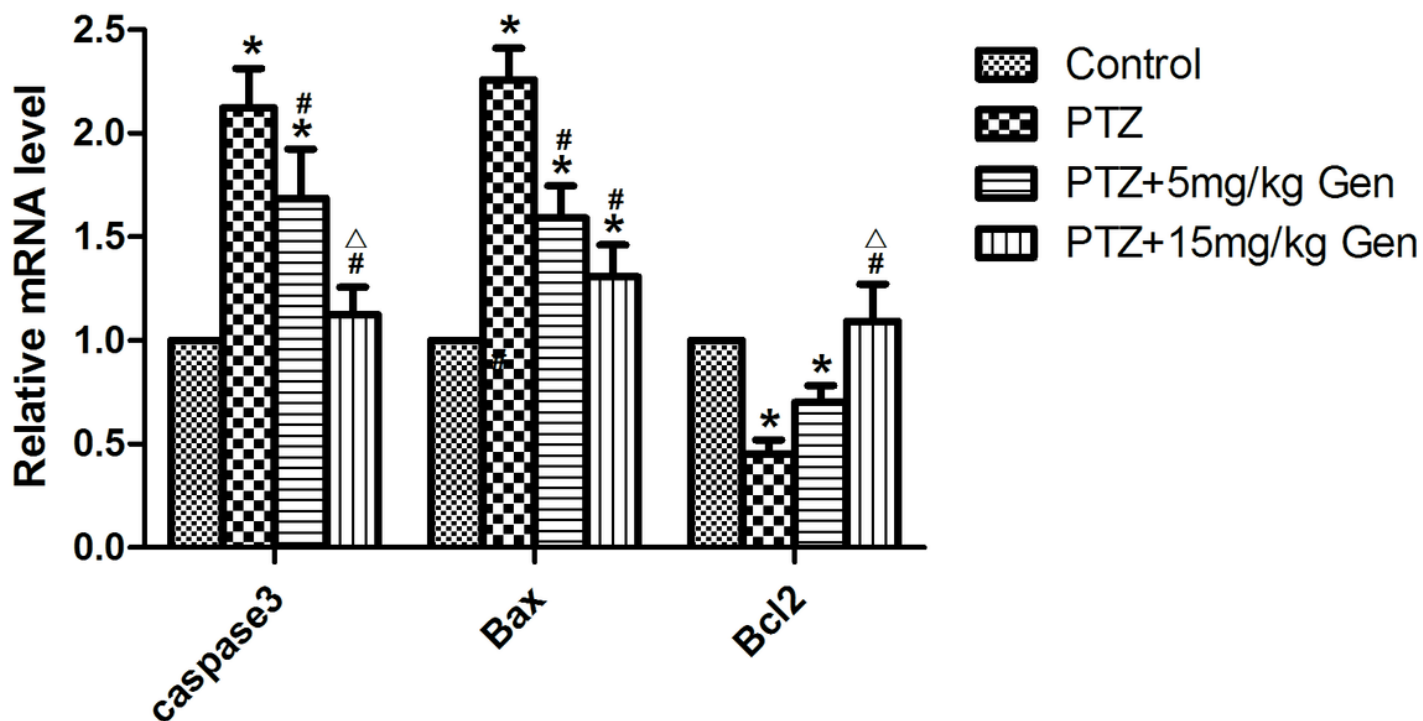


Figure 12

mRNA levels of caspase3, Bax and Bcl2 were measured after PTZ-induced epilepsy. Measurement data were expressed as mean±SD (n=16). *P<0.01, PTZ group vs. Control group; #P<0.01, Genistein treated group (5, 15 mg/kg) vs. PTZ group; ΔP<0.01, PTZ+15mg/kg Gen group vs. PTZ+5mg/kg Gen group.