Article

Discovery Medicine 2025; 37(195): 715–726
https://doi.org/10.24976/Discov.Med.202537195.62

Protective Role of Cerium Oxide Nanoparticle Pretreatment in Preventing Pilocarpine-Induced Epileptic Seizures

Dan Yang[1,2,†], Shuangfeng Yang[3,†], Jingya Deng[1,2], Xiyue Fan[1,2], Guohui Jiang[1,2], Fei Yang[1,2,*], Xiaoming Wang[1,2,*]

[1]Department of Neurology, Affiliated Hospital of North Sichuan Medical College, 637000 Nanchong, Sichuan, China
[2]Institute of Neurological Diseases, North Sichuan Medical College, 637000 Nanchong, Sichuan, China
[3]Department of Neurology, The People's Hospital of Yuechi County, 638300 Guangan, Sichuan, China
*Correspondence: yangfei5113@163.com (Fei Yang); wangnsmc@163.com (Xiaoming Wang)
[†]These authors contributed equally.
Published: 20 April 2025

Background: Epilepsy is a prevalent neurological disorder characterized by transient brain dysfunction due to abnormal neuronal discharges. Oxidative stress is strongly correlated with epilepsy onset and progression and is a critical factor in triggering seizures. Therefore, antioxidants may serve as effective anti-seizure treatments. Cerium oxide nanoparticles (CNP), which have antioxidant properties and function as nano-enzymes, may offer neuroprotective and therapeutic benefits for epilepsy. This study aims to investigate the effects of CNP on epilepsy.

Methods: We established a pilocarpine (PILO)-induced epilepsy rat model to assess the effects of pretreatment with different doses of CNP on epileptic behavioral changes, electroencephalographic activity, and nuclear factor erythroid 2-related factor 2 (NRF2) signaling in rats.

Results: In brief, a dose of 2.5 mg/kg CNP prolonged the latency of PILO-induced seizures in rats ($p < 0.05$), reduced the severity of seizures ($p < 0.05$), and decreased the 24-h mortality rate ($p < 0.01$). Additionally, CNP also extended the latency of epileptiform discharges ($p < 0.01$) and significantly decreased the average energy density of electroencephalographic activity ($p < 0.0001$). It inhibited seizure-induced lipid peroxidation ($p < 0.001$) and increased superoxide dismutase ($p < 0.05$) and catalase activities ($p < 0.01$). Furthermore, pretreatment with CNP elevated the expression of NRF2 and NADPH:quinone oxidoreductase 1 (NQO1) in antioxidative stress pathways ($p < 0.05$) and reduced neuronal necrosis and degeneration in CA1 and CA3 regions ($p < 0.05$).

Conclusions: CNP exhibits anti-epileptic and neuroprotective effects in PILO-induced epilepsy. This protective effect is likely due to the enhancement of the NRF2 signaling pathway, which regulates antioxidant enzymes, improves neuronal defense mechanisms against oxidative stress, and reduces seizure-induced neuronal damage.

Keywords: epilepsy; oxidative stress; cerium oxide nanoparticles; antioxidant; local field potentials

Introduction

Epilepsy is a prevalent neurological disease involving abnormal hypersynchronous neuronal discharge from neurons, resulting in periodic transient disturbances in brain function, and it affects approximately 50 million people worldwide [1]. Despite significant advances in the treatment of this condition, including neuromodulation and antiepileptic drugs, nearly 30% of epileptic seizures cannot be effectively controlled after standard therapy. Patients go on to develop refractory epilepsy, which not only causes severe psychological burdens for patients and families but also imposes significant economic strains on healthcare facilities and local communities [2–4]. The pathology of epilepsy is very complicated, and studies have shown that oxidative stress is correlated with its onset and progression [5–7]. Oxidative stress is caused by an imbalance between oxidation and antioxidation within the body, leading to decreased production of antioxidants and reduced scavenging of reactive oxygen species (ROS) [8]. During seizure, abnormal neuronal discharge and excitatory cell toxicity cause cells to produce excessive ROS, disrupting the intracellular redox balance, damaging neuronal structure and function, and ultimately causing neuronal degeneration and death [9–11]. It has been suggested that antioxidants may be an effective means of treating seizures.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a crucial transcriptional factor governing the balance between oxidation and antioxidation [12–14]. When cells are subjected to oxidative stress, NRF2 in the cytoplasm is activated and translocated to the nucleus, where it binds to antioxidant response elements (AREs) to initi-

ate the expression of downstream antioxidant enzymes and protective proteins, including heme oxygenase 1 and NADPH:quinone oxidoreductase 1 (NQO1), thereby enhancing the cells' antioxidant defenses and shields them from oxidative stress-induced damage [15,16]. Numerous studies have demonstrated that the NRF2-ARE signaling pathway can prevent various neurodegenerative diseases and protect tissues from the effects of oxidative stress and excitatory cell toxicity, making it an important therapeutic target for these diseases [17–19]. Additionally, the NRF2-ARE signaling pathway has protective effects in animal models of amygdala ignition, along with pentylenetetrazol-, pilocarpine (PILO)-, and kainic acid-induced epilepsy [20–22].

With the rapid development of nanotechnology, cerium oxide nanoparticles (CNP), as one of the most promising metal oxide nanozymes, have gained enormous research momentum due to their small particle size and robust ROS scavenging properties [23,24]. CNP mimics the functions of catalase (CAT) and superoxide dismutase (SOD) through the reversible conversion of two valence cerium atoms on the surface, thereby scavenging excessive reactive oxygen and reactive nitrogen species from cells and protecting them from damage caused by oxidative stress [25]. Several studies have revealed that CNP exhibits neuroprotective effects and is used to treat neurological conditions such as stroke [26–29]. It accomplishes this by activating the NRF2-ARE signaling pathway, increasing the cells' antioxidant potential to resist oxidative stress, thereby volatilizing the protective effect [30,31].

While the neuroprotective effects of CNP have been demonstrated in various neurological disorders, their impact on epilepsy remains underexplored. This study addresses this gap by establishing a PILO-induced epilepsy rat model to investigate the effects of pretreatment with different doses of CNP on epileptic behavioral changes, electroencephalographic activity, and NRF2-ARE signaling in rats. The aim is to provide an experimental basis for using CNP as an anti-epileptic treatment.

Materials and Methods

Experimental Animals

Forty-six specific pathogen-free adult Sprague-Dawley male rats (age, 6–8 weeks; body weight, 180–200 g) were obtained from the Laboratory Animal Center of North Sichuan Medical College and housed in a specific pathogen-free environment (room temperature, 22–25 °C; relative humidity, 50–60%) with circadian rhythm room settings and unrestricted access to food and water. The Ethics Committee of North Sichuan Medical College authorized the use of all rats in this study (No. NSMC 2023(016)).

Preparation and Characterization of CNP

CNP was prepared as earlier described [32]. In brief, 2.17 g of cerium (III) nitrate hexahydrate was dissolved in 50 mL of absolute ethanol. The solution was vigorously stirred for approximately 10 min, then 30 mL of ammonium hydroxide was added and stirred overnight. Subsequently, nanoparticles were purified by washing with anhydrous ethanol (3 times), deionized water (3 times), and centrifuging. Finally, the yellow precipitate was dried at 60 °C for 24 h.

CNP structure was examined by transmission electron microscopy (TEM, JEM-F200, JEOL Ltd., Tokyo, Japan). X-ray photoelectron spectroscopy (XPS, Thermo-Scientific K-Alpha, Thermo Fisher Scientific, Waltham, MA, USA) and X-ray diffraction (XRD, Bruker D8 Advance, Bruker, Karlsruhe, Germany) were also performed. Image J software (image J 1.53e, National Institutes of Health, Bethesda, MD, USA) was used to measure the CNP size, and Origin software (Origin 8.5, Origin Lab Corp., Northampton, MA, USA) was used to plot the histogram.

Experimental Groups

The rats were divided randomly into four groups: (1) the control group, (2) the 1.25 mg/kg CNP group, (3) the 2.5 mg/kg CNP group, and (4) the 5 mg/kg CNP group (n = 10 in each group). As previously reported [33,34], rats were pretreated with CNP for 10 days before the administration of PILO, once a day, by intraperitoneal injection (i.p.), and PILO was given 1 h after the last intervention to induce epilepsy.

Animal Epilepsy Model

The PILO model, which mimics most seizure types, was used [35]. In brief, rats were treated with lithium chloride (127 mg/kg, ip, CAS85144-11-2, Sigma-Aldrich, Darmstadt, Germany) for 20 h, followed by atropine (1 mg/kg, ip, H31021172, Hefeng, Shanghai, China) and PILO (35 mg/kg, ip, HY-B0726, MedChemExpress, Monmouth Junction, NJ, USA) 30 min later. If the rats failed to undergo seizure within 30 min of PILO administration, 20% of the initial dosage was administered at 10-min intervals until a grade 4 or 5 seizure occurred. Each animal received a maximum of 5 doses of PILO. After an epileptic seizure that lasted for 1 h, diazepam (10 mg/kg, ip, H31021864, Xudong Haipu, Shanghai, Chian) was administered. The standard criteria established by Racine [36] were used to score the seizure activity. Seizure activity in rats was assessed using the Racine score at 10-min intervals over a 60-min period, beginning when the seizure reached a severity of grade 4 or 5. Only rats experiencing recurring seizures of grade 4 or 5 were used in the subsequent analyses.

Surgical Procedures and Electrophysiological Recordings

Based on the behavioral outcomes, we employed electroencephalography to ascertain the impact of the optimal dosage of the CNP group compared to the control group on seizures caused by PILO in rats (n = 3 in each group). One week before recording electroencephalographic activity, rats were anesthetized by continuous inhalation of 5% isoflurane through a face mask for general anesthesia (MSS-3S, Renyi, Shanghai, China) and subsequently placed into a brain stereotactic device. A bone window was performed at the right hippocampus (the anterior fontanelle as the origin of the coordinate, 3.6 mm behind the anterior fontanelle, 2.8 mm on the right side of the sagittal suture, and 3.5 mm deep), and a 16-channel linear microelectrode array was implanted [37]. A Neural Data Acquisition System (OmniPlex, Plexon, Dallas, TX, USA) was used to preamplify ($\times$1000), filter (0.1–1000 Hz), and digitized at 4 kHz the local field potentials (LFPs). All of the electrophysiological recordings referred to the two ground screws and were conducted using direct current-coupled head stages.

Analysis of Electrophysiological Data

An epileptiform discharge event was defined as a minimum sustained duration of 5 seconds with a frequency greater than 5 Hz and a high amplitude greater than twice the baseline [38]. Based on previous studies [39,40], neurophysiological data were analyzed using NeuroExplorer software (NeuroExplorer® V5.0, Plexon, Dallas, TX, USA) to calculate the power spectra of β waves (13–30 Hz), as well as high-frequency oscillations (80–200 Hz), and the average energy density of the electroencephalography during the epilepsy modeling process. Image processing and statistical analysis were performed using MATLAB software (v7.1, MathWorks, Natick, MA, USA) and Prism software (Prism 6.0, GraphPad Software, Santiago, MN, USA), respectively.

Estimation of Oxidative Stress Markers

Determination of Malondialdehyde Concentrations

Approximately 24 h after the successful induction of epileptic seizures, rats (n = 3 per group) were continuously anesthetized with 5% isoflurane, limbs were fixed, and brain tissue was collected for pathological analysis after cardiac perfusion. The remaining rats, including those used to record electroencephalography, were sacrificed by rapid cervical dislocation, and the hippocampus was removed for oxidative stress marker analysis and western blotting. The hippocampus was combined with phosphate-buffered saline (PBS) at a weight-to-volume ratio of 1:9, homogenized, and centrifuged for 10 min at 10,000 $\times g$. The supernatant was used to assay the concentrations of malondialdehyde (MDA) and SOD. A portion of the supernatant was used for protein concentration determination. The concentration of MDA in the hippocampal homogenate was determined by an MDA Colorimetric Assay Kit (E-BC-K025-M, Elabscience, Wuhan, China). The substrate-supernatant mixture was centrifuged at 9569 $\times g$ for 10 min, and the optical density was measured at 532 nm using a microplate reader (Varioskan LUX, Thermo Fisher Scientific). The results were expressed as µmol/g protein.

Evaluation of SOD Activities

SOD activity was measured using a SOD Activity Assay Kit (E-BC-K019-M, Elabscience). After incubation at room temperature for 10 min, the optical density of the substrate-supernatant mixture was measured at 550 nm. SOD activity was expressed as U/mg protein.

Measurement of CAT Activity

The hippocampal tissue was combined with PBS at a weight-to-volume ratio of 1:10, homogenized, and centrifuged for 10 min at 8000 $\times g$ and 4 °C. The supernatant was used to measure CAT activity. The substrate and supernatant were combined and incubated, and the initial absorbance (A1) at 240 nm and the absorbance (A2) after 1 min were recorded. CAT activity was calculated using the formula $\Delta A = A1 - A2$ and CAT activity was expressed as U/mg protein.

Western Blotting

Hippocampal tissues were homogenized using lysis buffer supplemented with phosphatase and protease inhibitors (P1081, P1010, Beyotime, Shanghai, China), centrifuged, and the supernatant was separated. The total protein concentration was calculated by operating the BCA protein assay (Prod#23225, Thermo Fisher Scientific). In brief, 30 µg of the total protein samples were added to each lane. Electrophoresis was performed on a sodium dodecyl polyacrylamide gel (5% spacer gel, 10% separating gel) and subsequently electrotransferred to a polyvinylidene fluoride membrane (No.03010040001 Millipore, Burlington, MA, USA). The membranes were sealed with 5% non-fat milk for 1 h at room temperature, followed by overnight incubation at 4 °C with rabbit anti-NRF2 (1:6000, Cat 16396-1-AP, Proteintech, Wuhan, China), mouse anti-NQO1 (1:5000, Cat67240-1-1g, Proteintech), or rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000, AF7021, Affinity, Wuhan, China) antibodies. After washing with tris buffered saline with Tween 20, the membranes were incubated for 1 h with either goat anti-mouse IgG (1:5000, A0216, Beyotime) or goat anti-rabbit IgG (1:10,000, S0001, Affinity). An enhanced chemiluminescent system (Gel Doc XR+, Bio-Rad, Hercules, CA, USA) was used to observe the protein bands using a chemiluminescent kit (KF8003, Affinity). The gray intensity of protein bands was quantified using Image J software.

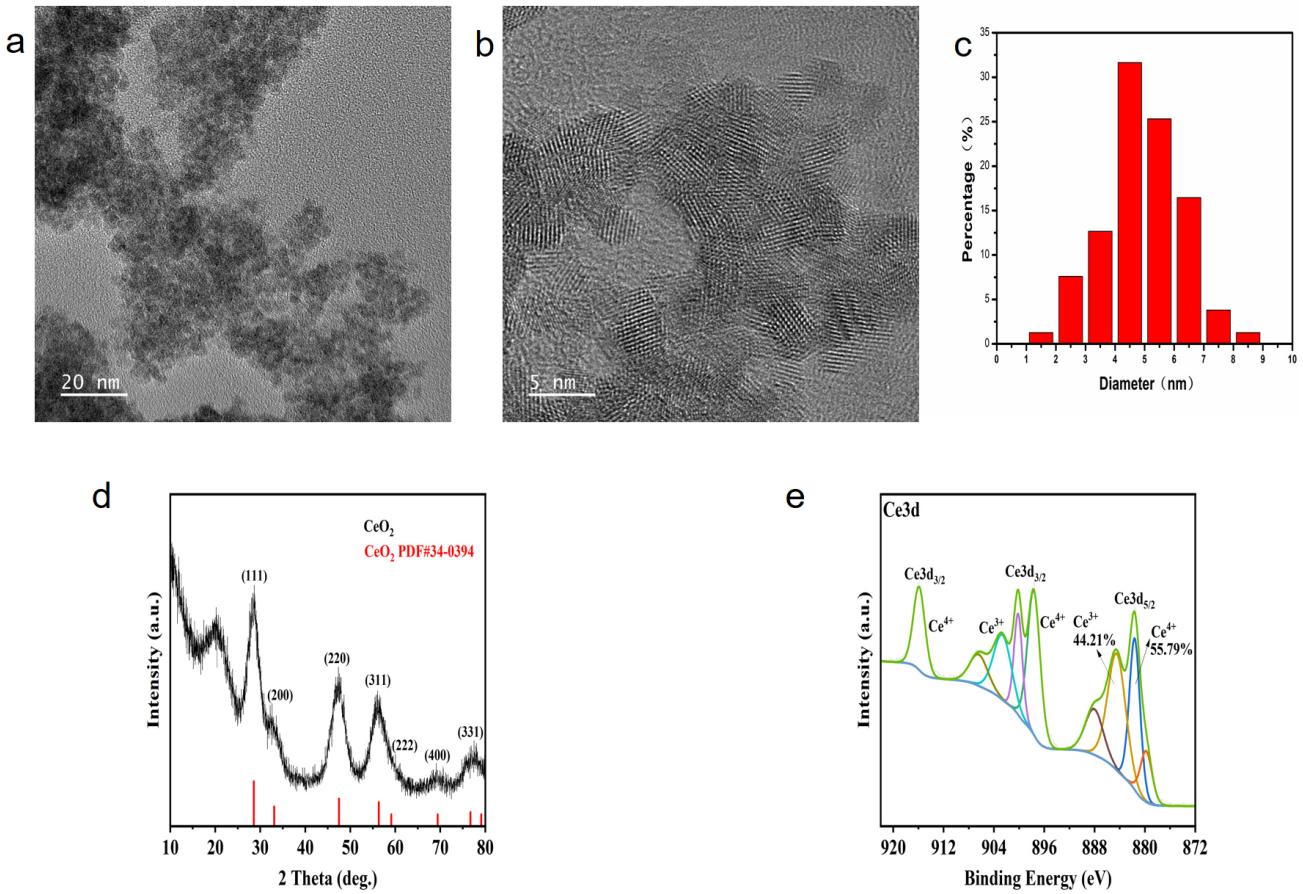

Fig. 1. Characterization of cerium oxide nanoparticles (CNP). (a,b) Transmission electron microscopy (TEM) images of CNP. (c) Histogram of CNP particle size. (d) X-ray diffraction (XRD) of CNP. (e) X-ray photoelectron spectroscopy (XPS) analyses of CNP.

Nissl Staining

Rat brains were removed and placed in 4% paraformaldehyde for 24 h, dehydrated, embedded in paraffin, and sliced (5-µm thick). Next, sections were baked at 60 °C for 1 h and stained using the method described for toluidine blue staining (BC0205, Servicebio, Beijing, China). Images were obtained with an Olympus microscope (Olympus, BX53-LED, Tokyo, Japan) at original magnifications of ×400. The neuron-counting procedure was carried out using Image J software. Three relative coronal plane slices (CA1 and CA3) in the hippocampal region of each brain were analyzed separately, and the average number of the three slices was used as the data for each sample. Finally, the brains of three rats from each experimental group were analyzed.

Hematoxylin-Eosin Staining

Hearts, livers, spleens, lungs, and kidneys were removed and immersed at room temperature in 4% paraformaldehyde for 24 h, dehydrated, and embedded in paraffin. Next, 5-µm-thick paraffin sections were prepared using a microtome (Thermohm3555, Thermo Fisher Scientific), attached to a glass slide coated with polylysine, and then dried in a constant temperature oven (MQD-S2R, Minquan, Shanghai, China) at 60 °C for 1 h. Sections were stained with hematoxylin and eosin (CAS517-28-2, Servicebio) and sealed with neutral resin. Images were captured using an Olympus microscope with an original magnification of ×100.

Statistical Analysis

SPSS software (SPSS 29.0, IBM, Chicago, IL, USA), Prism 6.0 software, and Adobe Photoshop software (CS6, Adobe Systems, San Jose, CA, USA) were used for statistical analysis and figure preparation. Quantitative data are expressed as mean ± standard error of the mean. The *t*-test or one-way analysis of variance was used to compare quantitative information between two or more normally distributed groups. The Mann-Whitney U test was adopted to compare the quantitative data of skewed distribution between the two groups, and the Bonferroni test was performed to compare the differences between groups after a one-way Analysis of variance. The Kruskal-Wallis test was used to compare the categorical data across multiple groups. In contrast, the chi-square or Fisher's exact test was used to compare the rates among various groups. $p < 0.05$ was considered a statistically significant difference.

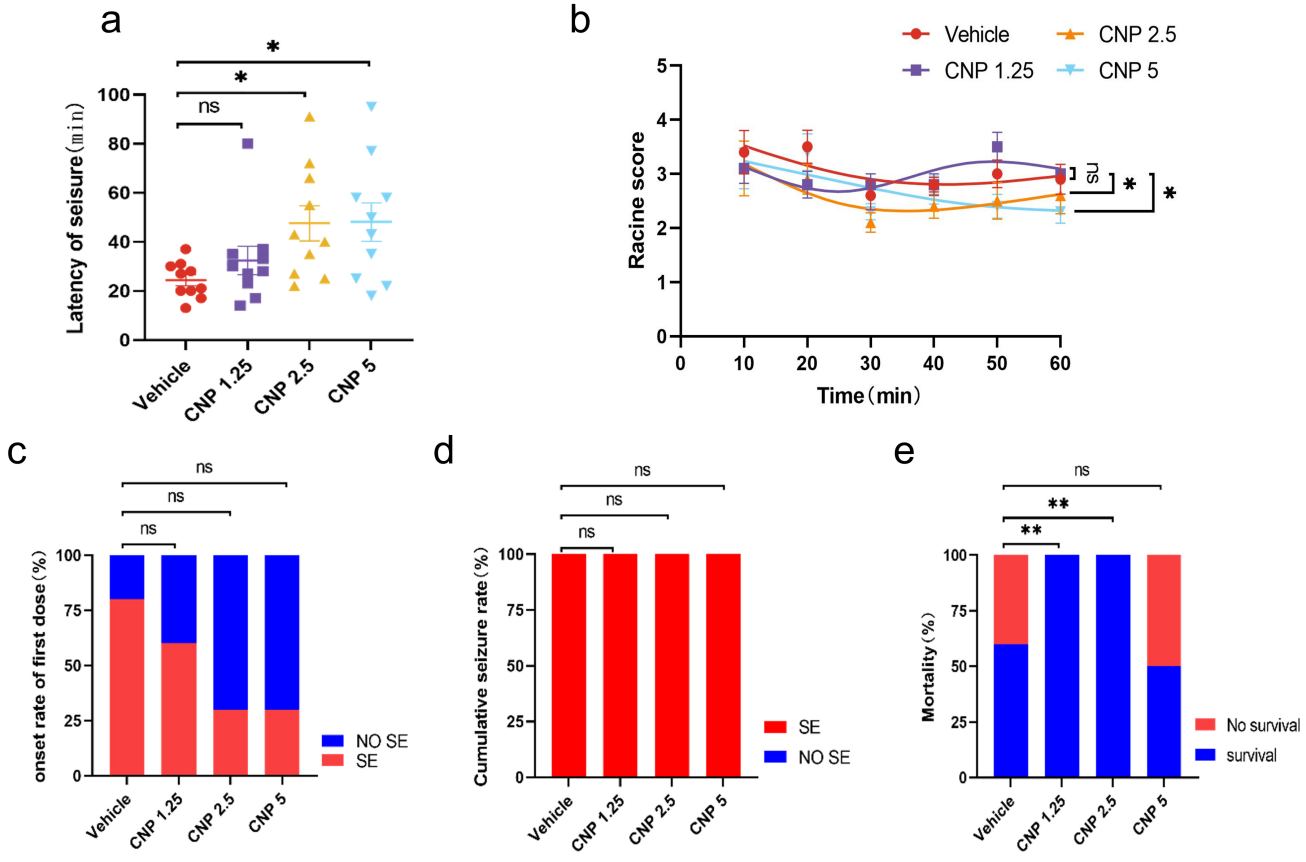

Fig. 2. Effect of various CNP doses against pilocarpine (PILO)-induced epilepsy in rats. (a) 2.5 mg/kg CNP prolonged the latency of the seizure. (b) 2.5 mg/kg CNP significantly reduced the severity of seizures (0 represents the onset of the first seizure at a Racine grade of 4 or 5). (c) Rate of seizures starting at grade 4 or 5 after the first dose of PILO. (d) Cumulative rate of grade 4 or 5 seizures in rats using PILO. (e) Effects of various CNP doses on the mortality rate 24 h after PILO-induced epilepsy in rats. Bars represent mean ± Standard Error of Means (SEM) (n = 10 for each group). *$p < 0.05$ and **$p < 0.01$ compared to the control group. ns, not significant.

Results

Characterization of CNP

CNP was synthesized as previously described and characterized by TEM, XRD, and XPS. As shown in Fig. 1a–c, the size of the synthesized CNP showed an average particle size of approximately 5 nm, and TEM indicated a relatively uniform spherical particle size of approximately 5 nm, consistent with an earlier study [32]. As shown in Fig. 1d, the peaks of the synthesized CNP at 28.61°, 47.59°, and 56.48° corresponded to the (111), (220), and (311) lattice planes, respectively, which corresponded to cerium oxide standard (cat no. 34-0394), confirming the formation of cerium oxide crystals. XPS showed that Ce^{3+} and Ce^{4+} coexisted on the surface of the synthesized CNP, and the ratios were 44.21% and 55.79%, which provided a chemical basis for the catalytic activity (Fig. 1e). Thus, we verified that CNP was successfully synthesized.

CNP (2.5 mg/kg) Considerably Reduces the Severity of Epileptic Seizures and Deaths During PILO-Induced Epilepsy

We observed the effects of different doses of CNP on the latency, severity, and 24-h mortality of PILO-induced epilepsy in rats (Fig. 2). Compared with the control group, CNP at 2.5 mg/kg and 5 mg/kg could prolong the latency of PILO-induced seizures ($p < 0.05$). At the same time, there was no obvious difference in the latency of seizures compared to the control and 1.25 mg/kg CNP groups ($p > 0.05$) (Fig. 2a). To verify the effect of different doses of CNP on seizure severity, the severity of seizures at 1 h was recorded and analyzed from the first occurrence of grade 4 or 5 seizures in rats (Fig. 2b). The outcomes showed that, compared to the control group, CNP at 2.5 mg/kg and 5 mg/kg reduced the severity of seizures ($p < 0.05$). In contrast, the 1.25 mg/kg CNP group exhibited no significant difference compared to the control group ($p > 0.05$). We also recorded and compared the first-dose seizure rate, cumulative seizure rate, and 24-h mortality rate of PILO-induced seizures in rats (Fig. 2c–e). No significant differences in the first-dose and cumulative seizure rates were

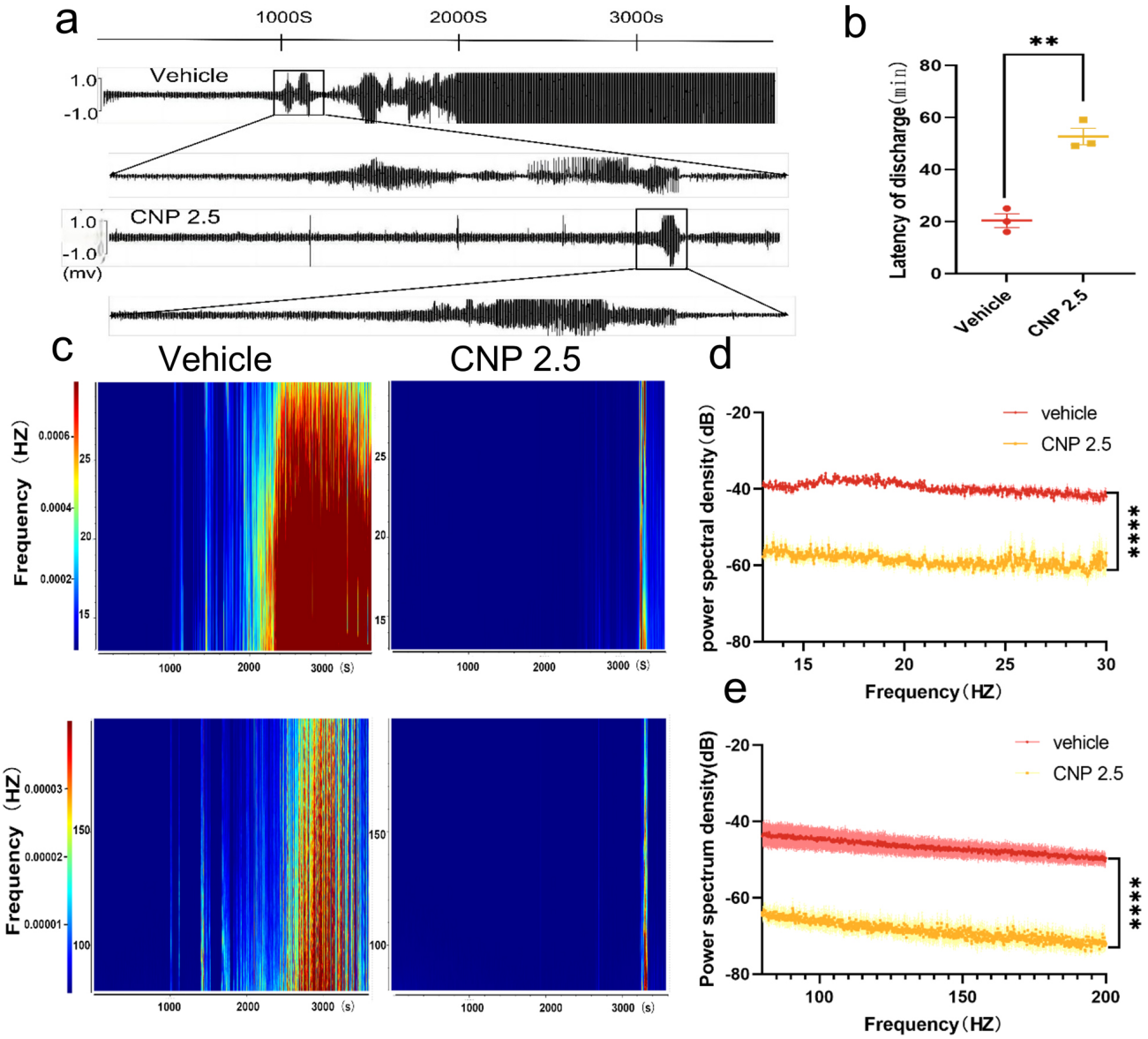

Fig. 3. Impact of CNP on epileptiform discharge from the hippocampus during PILO-induced seizures. (a) Effect of 2.5 mg/kg CNP on hippocampal electrical activity (the X-axis is 60 min, and the Y-axis is ± 1.0 millivolts). (b) 2.5 mg/kg CNP prolonged the latency of epileptic discharge. (c) Corresponding power spectrograms of each group (Colored bands show energy density; higher energy density is shown by redder bands). (d,e) 2.5 mg/kg CNP reduced brain electrical activity's mean energy density value. Data are shown as mean ± SEM. $^{**}p < 0.01$ and $^{****}p < 0.0001$ compared to the control group; n = 3 each group.

observed among the 1.25 mg/kg CNP, 2.5 mg/kg CNP, and 5 mg/kg CNP groups compared to the control group ($p > 0.05$). Four of ten, zero of ten, zero of ten, and five of ten rats died in the control, 1.25 mg/kg CNP, 2.5 mg/kg CNP, and 5 mg/kg CNP groups, respectively. The results show that, compared with the control group, CNP at 1.25 mg/kg and 2.5 mg/kg reduced the 24-h mortality rate after seizures ($p < 0.01$). In contrast, there was no statistical difference between the 5 mg/kg CNP and control groups ($p > 0.05$).

Impact of CNP on Epileptiform Discharge From the Hippocampus During PILO-Induced Seizures

Based on the above behavioral results, we screened the optimal dose of CNP for subsequent studies to further determine the role of CNP in epileptiform discharges. The electroencephalogram activities of rats injected with PILO for 1 h were recorded and analyzed (Fig. 3a,b). The results indicate that the latency of epileptiform discharges in the 2.5 mg/kg CNP group was significantly longer than that in the control group ($p < 0.01$).

We observed the effects of 2.5 mg/kg CNP on β-wave, power spectrum of high-frequency oscillation activ-

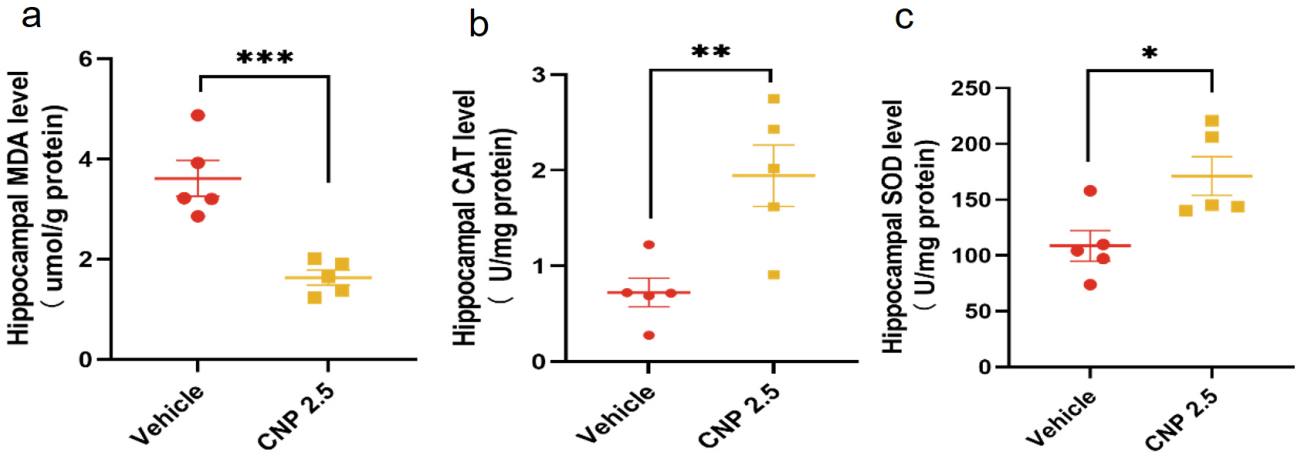

Fig. 4. Effect of CNP pretreatment on oxidative stress indexes. (a) Malondialdehyde (MDA) concentrations in the hippocampus of rats. (b) Catalase (CAT) activity in the hippocampus of rats. (c) Superoxide dismutase (SOD) activity in the hippocampus of rats. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$and ***$p < 0.001$ compared to the control group; n = 5 each group.

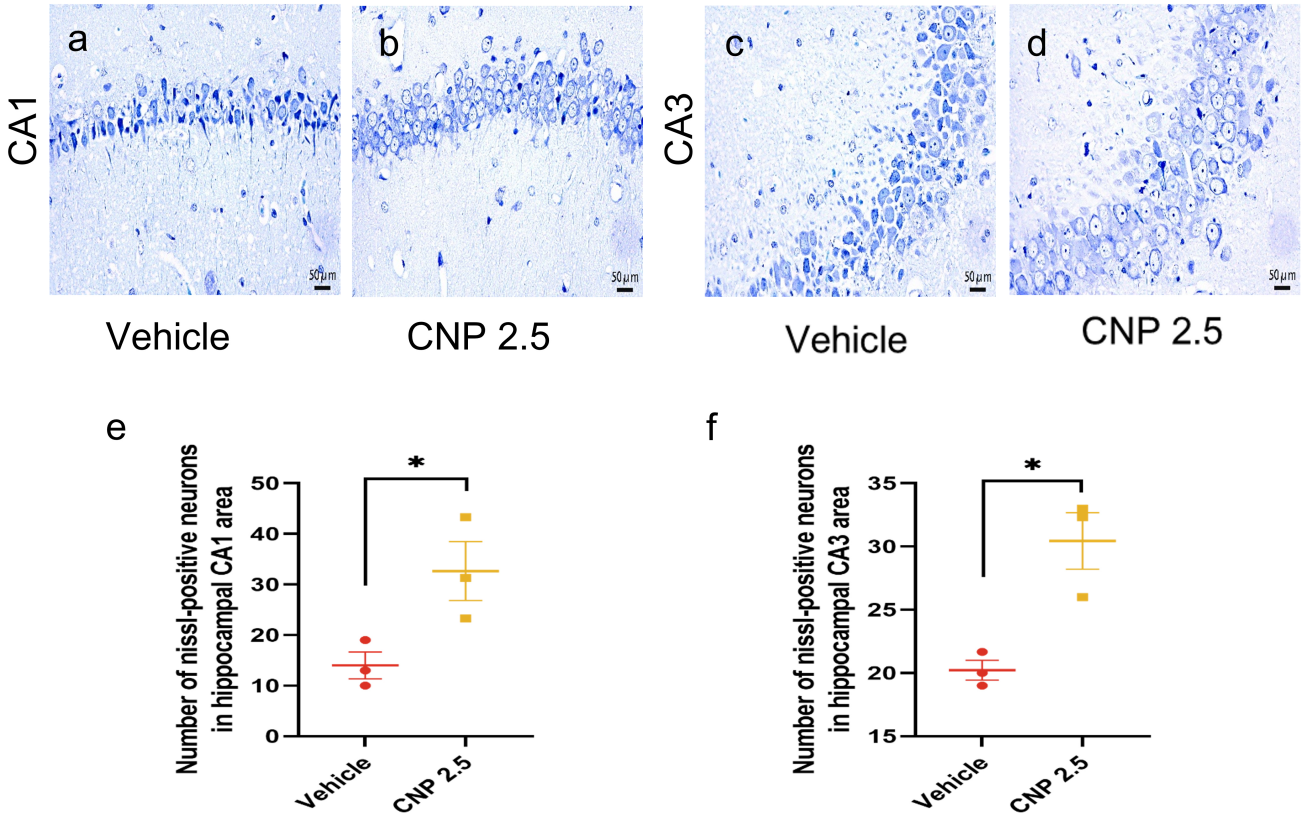

Fig. 5. Effect of CNP on epileptic seizure-induced hippocampal neurons detected by Nissl staining. Light microscopy of hippocampal CA1 area in the control group (a) and the 2.5 mg/kg CNP group (b). Light microscopy of hippocampal CA3 area in the control group (c) and the 2.5 mg/kg CNP group (d). Number of Nissl-positive neurons in hippocampal CA1 area (e) and CA3 area (f). Scale bar = 50 μm. Data are shown as mean ± SEM. *$p < 0.05$ compared to the control group; n = 3 each group.

ity, and average energy density of electroencephalogram in the hippocampus of PILO-induced epileptiform discharges to study the underlying electrophysiological mechanism of CNP on epileptiform discharges. The energy spectrum and energy density values of β-wave and high-frequency oscillation observed in the 2.5 mg/kg CNP group were notably lower than those in the control group ($p < 0.0001$) (Fig. 3c–e).

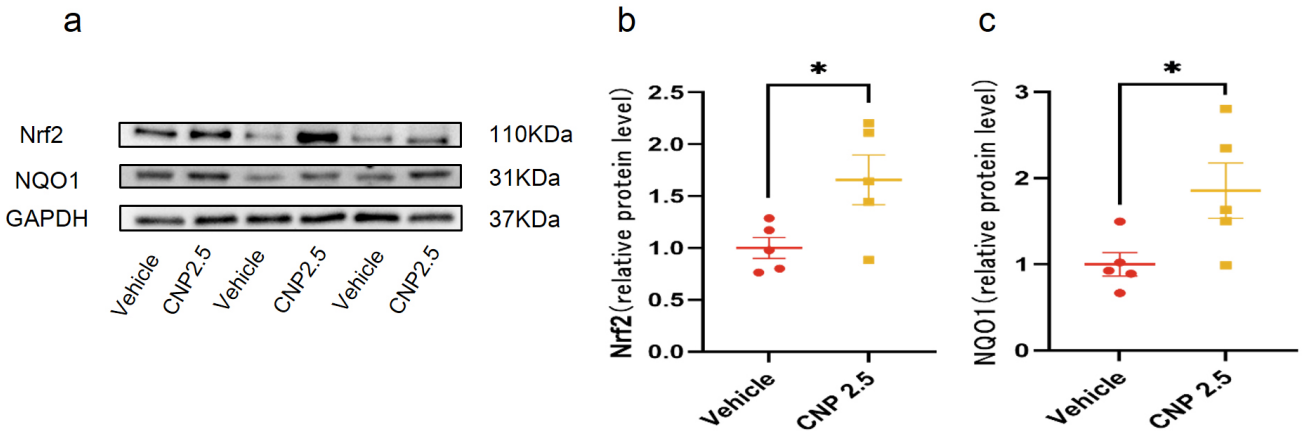

Fig. 6. Effect of CNP on the expression of erythroid 2-related factor 2 (NRF2) and NADPH:quinone oxidoreductase 1 (NQO1) in the hippocampus of rats. (a) Representative western blotting of NRF2 and NQO1 in the control and 2.5 mg/kg CNP groups. (b,c) The NRF2 and NQO1 levels in the Control and 2.5 mg/kg CNP groups. Data are shown as mean ± SEM. *$p < 0.05$ compared to the control group; n = 5 each group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Effect of CNP Pretreatment on Oxidative Stress Indexes

Oxidative stress is one of the key factors in the onset and progression of epileptic seizures. In this study, MDA, SOD, and CAT kits were used to examine the effect of CNP on oxidative stress indexes in the hippocampus of epileptic rats: it aimed to explore the relationship between oxidative stress, CNP, and seizures. Compared with the control group, the MDA content in the hippocampus of the 2.5 mg/kg CNP group was significantly decreased ($p < 0.001$). In contrast, CAT ($p < 0.01$) and SOD ($p < 0.05$) activities were significantly increased (Fig. 4). The results suggest that the expression of hippocampus antioxidant enzymes in the control group was significantly reduced, and the lipid oxidation products were significantly elevated. At the same time, the 2.5 mg/kg CNP pretreatment enhanced the activity of antioxidant enzymes and inhibited lipid peroxidation (LPO) in the hippocampus of epileptic rats.

Outcomes on Hippocampal Neurons of CNP

Seizures induce neuronal degeneration and death. Nissl staining was conducted on the brain tissues of the control group and 2.5 mg/kg CNP group to observe the neuronal morphology and count of Nissl bodies in CA1 and CA3 regions to evaluate the morphology of neurons. In Fig. 5, the neurons in the control group hippocampus were sparse, disordered, the number was reduced, the Nissl bodies were dissolved or disappeared, and the nuclei were shrunk or dissolved. In contrast, the neurons in the hippocampus of rats in the 2.5 mg/kg CNP group were relatively complete and dense, with relatively neat arrangement, abundant Nissl bodies, clear nuclei, and only less structural loss and vacuolar degeneration. Statistical analysis showed that the number of Nissl-positive cells in the CA1 and CA3 regions of the control group was lower than that of the 2.5 mg/kg CNP group ($p < 0.05$). The results indicated that CNP pretreatment can improve neuronal degeneration and necrosis induced by seizures.

CNP Upregulate NRF2, NADPH:Quinone Oxidoreductase-1 (NQO1) Expression

Western blotting was performed to further explore the effect of CNP on the levels of NRF2-ARE signaling pathway-related proteins in the hippocampus of epileptic rats. As shown in Fig. 6, the expression of NRF2 and NQO1 was significantly elevated in the 2.5 mg/kg CNP group compared to the control group ($p < 0.05$), indicating that CNP pretreatment could increase the levels of antioxidant-related signaling proteins in the hippocampus of epileptic rats.

CNP does not Exhibit Toxicity or Cause Pathological Alterations in Rat Organs

Hematoxylin-eosin staining was used to examine heart, liver, spleen, lung, and kidney specimens of rats in the control and 2.5 mg/kg CNP groups. All the organs were evaluated for any toxic changes as well as the presence of any extraneous material deposits through detailed observation of cellular morphology, arrangement, quantity and tissue structure. The results showed no abnormal pathological changes in these organs in the 2.5 mg/kg CNP group compared with the control group (Fig. 7).

Discussion

In this study, we demonstrated that 2.5 mg/kg CNP prolonged the latency of PILO-induced seizures in rats, diminished the intensity of seizures, and decreased the 24-h mortality rate. Additionally, CNP significantly prolonged the latency of epileptiform discharges, decreased the average energy density of electroencephalographic activity, and

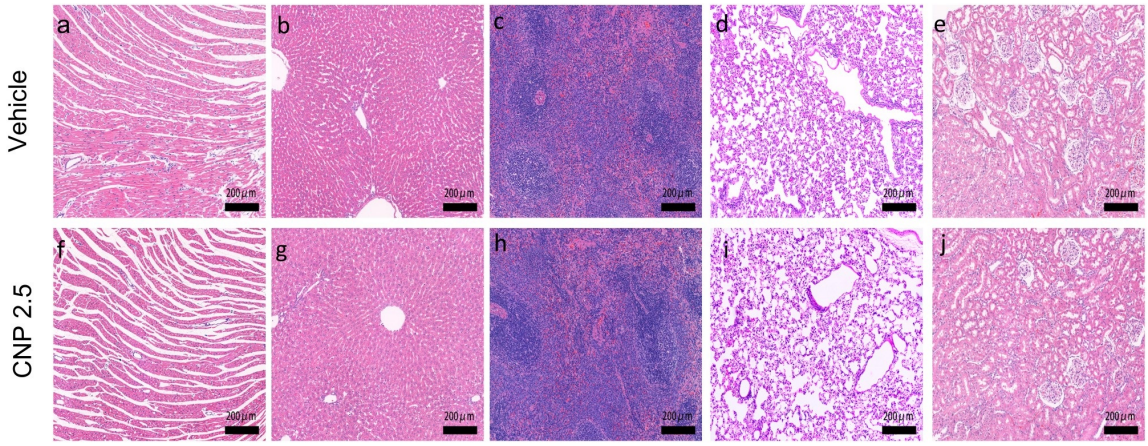

Fig. 7. CNP does not exhibit toxicity or cause pathological alterations in rat organs. Scale bar = 200 μm. (a–e) The control group heart, liver, spleen, lung, and kidney. (f–j) The 2.5 mg/kg CNP group heart, liver, spleen, lung, and kidney.

inhibited the increase in LPO-induced by seizures. It also increased SOD and CAT activities, reduced neuronal degeneration and necrosis in CA1 and CA3 regions, and elevated the expression of NRF2 and NQO1 in antioxidative stress pathways. These results indicate that CNP exerts neuronal protection and anti-epileptic effects in PILO-induced epileptic rats.

Epilepsy is a neurological disease that causes seizures due to abnormal neuronal activity [41]. Frequent seizures damage brain tissue and cause neuronal necrosis and degeneration, which subsequently results in alterations in glial cell proliferation, nerve fiber remodeling, and mossy fiber sprouting, leading to structural and functional plasticity changes in the central nervous system, which further aggravate the condition [42,43]. Oxidative stress, neuroinflammation, neurotransmitter imbalance, structural and functional abnormalities of cellular ion channels, and glial cell proliferation in the central nervous system underlie its pathogenesis, among which oxidative stress is considered the primary factor leading to seizures [44]. Oxidative stress during and after seizure aggravates mitochondrial dysfunction and increases seizure severity, resulting in neuronal injury and death [45]. Therefore, antioxidants may mitigate seizures and improve prognosis [46].

CNP, a biocompatible nanozyme, has multifunctional biomimetic activity [47]. Compared with other antioxidants, CNP has several advantages: small particle size, strong cell permeability, recyclable enzyme activity, and potent antioxidant activity [48]. The antioxidant activity of CNP is based on the redox sites provided by surface oxygen vacancies, reversible conversion of Ce^{3+}/Ce^{4+}, simulation of SOD and CAT activities, and removal of excessive ROS in a self-regenerative and energy-free manner [49]. To investigate whether CNP exhibits protective effects against seizures, rats were pretreated with different doses of CNP before seizures were induced with PILO. The results suggested that 2.5 mg/kg CNP prolonged the latency period of seizures and epileptiform discharges in rats and reduced the 24-h mortality rate, suggesting that CNP exhibits some degree of protection against PILO-induced seizures.

A previous study has shown that oxidative stress underlies the mechanism of seizure [6]. During seizures, excessive ROS exceeds the intracellular antioxidant capacity, leading to oxidative stress, causing LPO, protein oxidation, and DNA damage, ultimately leading to neuronal necrosis and degeneration [5,47]. As one of the most essential products of LPO in the cell membrane, MDA exacerbates cell membrane damage and is commonly used to assess the degree of oxidative metabolism in lipid membranes and indirectly assess the levels of free radicals generated by LPO and the degree of damage to tissue cells [50]. Numerous studies have found that LPO in the hippocampus increases during PILO-induced seizures [51,52]. Our results are consistent with these findings: we found that the level of MDA in the hippocampus of the control group was significantly higher than that of the 2.5 mg/kg CNP group, which may be caused by excessive ROS generation during seizures but may be scavenged by CNP as a powerful antioxidant. Therefore, we used CNP as an intervention agent to explore the possibility of preventing seizures and secondary damage by inhibiting oxidative stress.

Furthermore, many antioxidant enzymes can even prevent or repair the damage caused by oxidative stress and regulate the redox signaling pathway. The roles of CAT and SOD in regulating the cytotoxicity of superoxide anions have been reported [51]. Our findings suggest that the activities of CAT and SOD decreased significantly at 24 h after seizure. In contrast, pretreatment with 2.5 mg/kg CNP significantly enhanced the activities of SOD and CAT, suggesting that this increase may be related to the anti-epileptic effects of CNP. CNP pretreatment may also improve the antioxidant capacity of rats by curbing the increase in MDA levels and boosting SOD and CAT activities.

Previous studies have indicated that oxidative stress drives different forms of neuronal injury and neuronal death during seizures [47,53,54]. The death of hippocampal neurons facilitates epileptogenesis, seizures, and epilepsy [55]. However, antioxidants prevent hippocampal cell loss and reduce seizure frequency in epileptic rats [56]. In this study, CNP pretreatment inhibited the increase in MDA levels and the decrease in SOD and CAT activities. We used Nissl staining to analyze the neuronal morphology within the hippocampus of rats with epilepsy. The results indicated that the count of Nissl body-positive cells in the 2.5 mg/kg CNP group notably exceeded that in the control group, suggesting that CNP pretreatment can prevent seizure-induced damage to neurons.

NRF2 serves as the primary transcriptional factor and modulator of response to cellular oxidative stress, which regulates the expression of multiple antioxidant enzyme genes and proteins [20,57]. The NRF2-ARE signaling pathway is a critical target for treating epilepsy. Earlier studies have reported that oxidative stress initiates the NRF2-ARE signaling pathway at an early seizure stage [21,57]. Mazzuferi *et al*. [21] observed an upregulation in the NRF2 mRNA level in the hippocampus of epilepsy patients, a finding that was later confirmed in a mouse model of epilepsy. It was reported that NRF2, heme oxygenase 1, and NQO1 were gradually upregulated after a seizure, peaked at 72 h, and gradually decreased. Furthermore, Cao *et al*. [56] observed that the expression of NRF2 and NQO1 increased at 24 h after seizure but could not resist LPO, oxidative damage, and apoptosis caused by oxidative stress. After antioxidant pretreatment, the endogenous antioxidant proteins and phase II detoxification enzymes were significantly upregulated, which prevented apoptosis and induced a protective effect. As a potent antioxidant, CNP has been shown to enhance NRF2-ARE signaling to regulate the expression of antioxidant enzymes and inhibit oxidative stress-induced cellular injury and dysfunction [58,59]. This study confirmed that CNP pretreatment could upregulate the expression of NRF2 and NQO1 in the hippocampus, effectively inhibit the increase of LPO, and protect neurons from seizure-induced damage compared with the control group, suggesting that the antioxidant properties of CNP may be crucial in its protective effect on rats with epilepsy.

This study had some limitations. First, we only evaluated the effect of CNP on PILO-induced epileptic rats. Whether similar effects exist in other epilepsy models and chronic epilepsy remains to be explored. Second, although numerous studies have demonstrated that CNP has no apparent toxicity in the body and exhibits many beneficial effects, these data are from preclinical studies. Therefore, it is important to conduct further studies on the effects of cerium, a rare metal, on the body, especially its long-term effects.

Conclusions

This study revealed that CNP pretreatment has a potential protective effect against PILO-induced seizures. We demonstrated that CNP regulates the expression of antioxidant proteins by upregulating the NRF2-ARE signaling pathway, enhancing the defensive function of neurons against oxidative stress, and inhibiting oxidative stress-mediated neuronal necrosis and degeneration. Our findings suggest that CNP may have potential as an anti-epileptic treatment by enhancing antioxidant effects; however, its safety needs to be further explored.

Availability of Data and Materials

All data generated in this published article for further data are available from the corresponding authors.

Author Contributions

DY, SY, FY and XW—designed the research study; DY, SY, JD and XF—performed the research; GJ, FY and XW—provided help and advice on the animal experiment; DY and SY—analyzed the data. DY, SY drafted the manuscript. All authors were involved in the critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The protocols of animal experiments were in compliance with the Chinese Animal Welfare Act and were reviewed and approved by the Ethics Committee of North Sichuan Medical College (Nanchong, China). NO. NSMC 2023(016)).

Acknowledgment

Not applicable.

Funding

This study was supported by the 2023 University-level scientific research project of North Sichuan Medical College (CBY23-QDA29) and Nature Science Foundation of Sichuan Province (2023NSFSC0622).

Conflict of Interest

The authors declare no conflict of interest.

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