Article

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DARS2 Attenuates Pathophysiological Characteristics and Apoptosis in Temporal Lobe Epilepsy Rats by Targeting Nrf2 Pathway

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Background: Temporal lobe epilepsy (TLE) is a common form of epilepsy, characterized by recurrent seizures, hippocampal tissue cell loss, and cognitive impairment. This study aimed to investigate the role of aspartyl-tRNA synthetase 2 (*DARS2*) in regulating the pathophysiological characteristics and apoptosis of hippocampal tissue in a rat model of TLE.
Methods: TLE was induced in rats using pilocarpine. Gene and protein expression levels in hippocampal tissues were assessed by real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting, respectively. Spatial memory was evaluated using the Morris water maze test, and histopathological changes were analyzed by hematoxylin and eosin (HE) staining. The levels of inflammatory cytokines and antioxidant-related factors were determined by enzyme-linked immunosorbent assay (ELISA).
Results: DARS2 expression was significantly reduced in hippocampal tissues of TLE rats ($p < 0.05$). Upregulation of DARS2 improved spatial memory, alleviated histopathological damage, decreased cell apoptosis and inflammation, enhanced antioxidant capacity, increased expression of heme oxygenase-1 (HO-1) and B-cell lymphoma 2 (Bcl-2), and reduced cleaved caspase-3 levels ($p < 0.05$). Mechanistically, DARS2 promoted nuclear factor erythroid 2-related factor 2 (Nrf2) protein expression in rat hippocampal tissues, and silencing Nrf2 reversed the protective effects of DARS2 overexpression ($p < 0.05$).
Conclusion: DARS2 exerts neuroprotective effects in TLE by mitigating rat hippocampal pathophysiological damage, suppressing apoptosis, and enhancing antioxidant defenses through activation of the Nrf2 signaling pathway. These findings suggest that DARS2 may serve as a promising therapeutic target for the treatment of TLE and related cognitive deficits.

Keywords: epilepsy; aspartyl-tRNA synthetase 2; nuclear factor erythroid 2-related factor 2; antioxidant; inflammation

Introduction

Epilepsy is a prevalent neurological disease of the brain characterized by chronic recurrent episodes, but its underlying mechanisms are very complex and yet been fully elucidated [1–3]. A variety of factors, such as oxidative stress injury, glutamate excitotoxicity, and calcium overload, can lead to abnormal firing of neurons and trigger epilepsy [4]. The oxygen-free radical chain reaction produced by oxidative stress is the core pathological link of neuronal damage [5].

Mitochondrial oxidative stress injury refers to mitochondrial reactive oxygen generation and lipid peroxidation, accompanied by damage to the mitochondrial ultrastructure caused by activation of nicotinamideadenine dinucleotide phosphate oxidase (NADPH oxidase, Nox) [6–8]. Mitochondria are not only an important site for the production of free radicals after a seizure, but also a target of cellular stress [6]. At the same time, mitochondrial oxidative damage and dysfunction can increase the susceptibility to epilepsy and aggravate nerve damage caused by epilepsy [9]. nuclear factor erythroid 2-related factor 2 (*Nrf2*) plays a key role in the regulation of oxidative stress in cells [10,11]. In normal cells, mitochondrial membrane potential can be maintained through cellular respiration, but in neuron cells of *Nrf2* knockout mice, mitochondrial membrane potential and ATP synthesis are reduced, and the efficiency of oxidative phosphorylation is also reduced [12]. ATP generation mainly depends on the oxidative phosphorylation of mitochondria and the tricarboxylic acid cycle, of which Nox is an important part of the tricarboxylic acid cycle [13]. However, at present, the possible network regulatory mechanism of *Nrf2*/Nox in neural injury in rats with temporal lobe epilepsy is unclear and requires further research [14].

A growing body of research indicates that mitochondrial proteins play pivotal roles in disease development and cellular homeostasis [15–17]. For instance, comparative transcriptome analyses in ilocarpine-induced epileptic seizure models revealed hundreds of dysregulated mitochondrial-related genes [18]. Aspartyl-tRNA synthetase 2 (DARS2) is a mitochondrial enzyme essential for protein synthesis within mitochondria, and accumulating evidence shows that *DARS2* is involved in diverse biological processes, including cellular proliferation, regula-

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tion of apoptosis, and cancer progression [19,20]. *DARS2* has been reported to modulate mitochondrial function and oxidative stress responses, thus influencing cancer cell survival and resistance to therapy [21]. Moreover, *DARS2* has been shown in a previous report to prevent neuroinflammation and apoptotic neuronal loss [22]. Through the analysis of dataset GSE297566, we identified *DARS2* as a differentially expressed gene associated with Temporal lobe epilepsy (TLE). Previous study has shown that exposure to δ-aminolevulinic acid/iron induces *Nrf2*-mediated cytoprotection in *DARS2*-deficient fibroblasts with impaired energy and antioxidant metabolism [23]. Therefore, we selected *DARS2* as the focus of this study to investigate its biological role in a rat TLE model and to elucidate its potential regulatory mechanisms via the *Nrf2* pathway.

Materials and Methods

Animal and Modeling

96 male Sprague-Dawley rats (SD, 190–230 g) were purchased from Hangzhou Medical College (China). All male animals were housed in groups of four or five and maintained under standard laboratory conditions (12/12-h light/dark cycle, 22 ± 2 °C, 50%–60% humidity, and *ad libitum* to food and water). All animals were used in compliance with the Regulations on the Administration of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (MOST version), and approved by the Zhejiang Provincial Experimental Animal Center Animal Welfare and Ethics Committee [No. ZJCLA-IACUC-20011150] before the study began.

Nrf2-shRNA sequence targeting the *Nrf2* gene (5′-GCUCAGAACUGUAGGAAAA-3′, Horizon, USA) was inserted into the adeno-associated virus (AAV) construct. The construct was transfected into HEK-293 cells (HKb20, Anweisci, China) using a three-plasmid co-transfection method to generate AAV expressing *Nrf2*-shRNA (AAV-shNRF2). Scrambled shRNA (5′-TTCTCCGAACGTGTCACGT-3′) was used as a negative control (AAV-shNC). For *DARS2* overexpression, the pAAV-CMV-MCS was used as the shuttle vector, and the recombinant AAV vector (AAV-*DARS2*) was constructed using the three-plasmid co-transfection technique. The empty vector of AAV was used as a negative control (AAV-NC).

The experimental protocol was conducted in two sequential stages. Experiment 1: To investigate the effect of *DARS2* overexpression in TLE model rats, SD rats in this study were randomly divided into 4 groups (n = 12 each group). Control group: rats were intraperitoneally injected with 0.9% saline. TLE group: intraperitoneal (IP) injection of pilocarpine (117757, Sigma-Aldrich, Germany), hippocampal stereotactic injection of normal saline. TLE + NC group: injection (IP) of pilocarpine to make a TLE model, negative control (NC) was injected into the hippocampus through stereotactic positioning. TLE + *DARS2* group: injection (IP) of pilocarpine to make a TLE model, and *DARS2* overexpression plasmid was injected into the hippocampus.

Experiment 2: To investigate the effect of the *DARS2-Nrf2* pathway on TLE rats, the SD rats in this study were randomly divided into 4 groups (n = 12 each group). TLE group: intraperitoneal (IP) injection of pilocarpine, hippocampal stereotactic injection of normal saline. TLE + *DARS2* group: injection (IP) of pilocarpine to make a TLE model, and *DARS2* overexpression plasmid was injected into the hippocampus. TLE + shNC+ *DARS2* group: injection (IP) of pilocarpine to make a TLE model, and *DARS2* overexpression plasmid and shNC were injected into the hippocampus. TLE + sh*Nrf2*+ *DARS2* group: injection (IP) of pilocarpine to make a TLE model, and *DARS2* overexpression plasmid and sh*Nrf2* were injected into the hippocampus.

Except for the control group, all rats were injected with pilocarpine 20 mg/kg (117757, Sigma-Aldrich, Germany) for the first time 18–24 h after 3 mmol/kg lithium chloride (LiCl, 127 mg/kg, L4408, Sigma-Aldrich, Germany) intraperitoneally. Atropine sulfate (1 mg/kg, A800762, Macklin, China) was administered 30 minutes (min) before injection. 30 min after the first injection of pilocarpine, non-epileptic rats were injected with pilocarpine 10 mg/kg every 30 min until seizures [24]. After the injection of the maximum dose of 60 mg/kg, only a brief attack with Racine of grade III or less was not considered, and rats unable to model were eliminated. According to the Racine scale [25]: grade I, clustered whisker and facial movements with chewing motions; grade II, facial spasm with rhythmic nodding; grade III, unilateral forelimb clonus or tonus; grade IV, bilateral forelimb tonic-clonic seizures with rearing; and grade V, loss of posture or generalized tonic-clonic seizures (GTCSs) with a fall or death. One hour after the onset of status epilepticus, diazepam (10 mg/kg, i.p., DZ2, Hameln Pharmaceuticals ltd, UK) was administered to terminate seizures. If the epilepsy effect was not good, then a supplemental injection was given 30 min later to ensure complete termination of seizures. The surviving rats entered the quiet period after the acute phase and exhibited symptoms such as crouching, eating poorly, and irritability during the first 2–4 days. At this point, sucrose physiological saline (0.5 mL/100 g) was injected intraperitoneally daily to maintain the normal physiological state of the rats. Then, the behavior of the rat had become normal. After a rest period of 7 to 20 days, spontaneous epilepsy began to develop into chronic epilepsy. The rats were given 12 h video surveillance every day, and the records were viewed in time. Grade IV or above was used as a successful epilepsy model, and unsuccessful model animals were removed (with an approximately 90% success rate). Based on previous experience, 12 rats were subjected to the modeling procedure in each group, ensuring that 10 rats from each group would proceed to the subsequent experiments.

Table 1. All primers in this study.

ID	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
DARS2	CCATGACTTTTGCTGAGGCAT	TGACAGTTCCTIGGGGCTTAG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGCACTGTGGTCATGAG
Nrf2	TGTCAGCTACTCCCAGGTTG	ATCAGGGGTGGTGAAGACTG
HO-1	ACAGAAGAGGCTAAGACCG	CAGGCATCICCTTCCATT
Bcl-2	AGGATAACGGAGGCTGGGTA	GGGGAGCAAAGCCACAAACT

DARS2, aspartyl-tRNA synthetase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; Bcl-2, B-cell lymphoma 2.

Rats in the control group were injected (IP) with 0.9% physiological saline and observed for their behavior changes. Two weeks before modeling, the rats were anesthetized with 2% isoflurane. Subsequently, a stereotactic frame (Leksell Coordinate Frame G; Elekta Instruments, Sweden) was used to fix the anesthetized rats. The AAV solution (1×10^{13} vg/mL) was injected bilaterally into the hippocampus at a rate of 0.05 µL/min (total volume 2 µL). The coordinates of the four injection sites were as follows: (a) AP, –3.8 mm; ML, –1.5 mm; DV, –3.4 mm; (b) AP, –4.3 mm; ML, –3.0 mm; DV: –3.9 mm; (c) AP, –4.8 mm; ML, –5.2 mm; DV, –3.8 mm; (d) AP, –4.8 mm; ML, –5.2 mm; DV, –6.4 mm. Rats (n = 4) were deeply anesthetized by intraperitoneal injection of 3% sodium pentobarbital overdose (100 mg/kg, APExBIO, USA), followed by cardiac perfusion with saline at a rate of 4 mL/min. When the saline infusion was nearly complete, it was rapidly switched to pre-cooled 4% paraformaldehyde (PFA) solution. Next, the entire hippocampal tissue was isolated from the animal and immediately placed in fresh, pre-cooled 4% PFA for further fixation for 4 hours. After the fixation process was completed, the hippocampus was flash frozen in liquid nitrogen and stored at –80 °C for histological analysis. Furthermore, the remaining rats (n = 6) were euthanized by intraperitoneal injection of 3% sodium pentobarbital (150 mg/kg), and the tissue samples were retained for molecular analysis.

Real-time Quantitative PCR (RT-qPCR)

Total RNA was extracted from 30 mg of hippocampal tissue and approximately 10^6 nerve cells using the TRIzol method. It was worth noting that the RNA pellet should be handled on ice when dissolving. Reverse transcription reactions were used with a kit (N8080234, ThermoFisher, USA). cDNA and RNA concentrations were measured using a NanoDrop One/OneC ultra-micro UV spectrophotometer (840-317400, Thermo Scientific, MA) . RT-qPCR was detected using One Step TB Green PrimeScript RT-qPCR Kit (RR066A/B, TaKaRa, China) and detection system (ABI 7500, Life Technology, USA). The gene expression was calculated using $2^{-\Delta\Delta Ct}$ method [26]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control. All primers were listed in Table 1.

Morris Water Maze Test

Each group of rats was subjected to a Morris water maze test, which was conducted four days after the successful model establishment. The water maze (BW-MWM101, Shanghai Biowill Co., Ltd., China) consists of a circular pool and a video acquisition and analysis system. While keeping the spatial clue around the Morris water maze unchanged, an appropriate amount of ink was added to the circular pool to prevent rats from seeing the platform. The platform was fixed in the center of one quadrant of circular pool. Morris water maze experiment was performed for 7 days with dim indoor light and temperature of 23–25 °C. The first 6 days were the training period: the head of the animal was placed in the water wall by the pool, and the recording was stopped after the rat had reached the platform for 5 s. The longest recording time is specified as 60 s. If the rats cannot reach the stage within 60 s, they are guided to the platform by a thin stick for 10 s. The time to reach the platform is determined as the escape latency. On the 7th day, the platform was removed and the percentage of swimming time and distance in the target quadrant in relation to the total time and distance was measured as a result of spatial memory. The personnel responsible for evaluating the primary endpoints were blinded to the group allocation of the animals.

Hematoxylin-Eosin (HE) Staining

The hippocampus tissue of each group was fixed with 4% paraformaldehyde and then stained with conventional hematoxylin-eosin (C0105, Beyotime, China). The sections were put into xylene (A530011-0500, Bioengineering, China), gradient alcohol hydration (64-17-5, Nanjing Reagent, China). Finally, the sections were washed with distilled water. Then the sections were stained with hematoxylin for 7 min, washed with tap water, stained with eosin for 1 min, hydrated with gradient ethanol (100%, 90%, 80%, 70%) for 1 min, and then dried and sealed with neutral gum. Histopathological changes were observed under a microscope (200×, CKX53, OLYMPUS, Japan), and recorded by a photo.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

To observe the apoptosis of hippocampal tissue cells in each group, TUNEL staining was performed using the TUNEL kit (11684795910, Roche, Switzerland). Tissue sections were routinely dewaxed into water and incubated with proteinase K (20 mg/L) for 15 min at room temperature. The sections were then added to the TUNEL solution in the dark at 37 °C and incubated for 60 min. Finally, the sections were lightly stained at 37 °C and mildly counterstained with hematoxylin for 5–30 min, washed and sealed with neutral balm. Finally, the sections were analyzed under fluorescence microscope (100×, CKX53, OLYMPUS, Japan), and TUNEL-positive (red) cells were counted using a blind method. Quantitative statistics were performed using the ImageJ software (Version 1.54k, Bio-Rad, USA).

Determination of Inflammatory Factors and Oxidant/nitro Oxidation State

The hippocampal tissue was removed from the liquid nitrogen tank. Each group of samples was diluted and homogenized with a certain proportion of normal saline on ice. The tissue was centrifuged at 4 °C for 20 min (3000 g). Expression of tumor necrosis factor α (TNF-α, PT516, Beyotime, China), interleukin-1β (IL-1β, PI303, Beyotime, China), and interleukin-6 (IL-6, PI328, Beyotime, China) in the hippocampal tissue supernatant was detected by enzyme-linked immunosorbent assay (ELISA). The kits were used to detect superoxide dismutase (SOD, CB10258-Ra, Coibo, China), glutathione (GSH, CB10393-Ra, Coibo, China), lipid permaldialdehyde (MDA, ml077384, Mlbio, China), and total NOx (RX2D389296, Ruixinbio, China) content in hippocampal tissue, respectively. The optical density (OD) was measured at a wavelength of 450 nm using a microplate reader (AMG-2202-025, Bio-Tek, USA).

Western Blotting Analysis

The extraction of total protein from hippocampal tissues was performed by 150 µL radioimmunoprecipitation assay (RIPA) lysis buffer (P0013, Beyotime, China) containing protease phosphatase inhibitor mixture (P1045, Beyotime, China). The detection of protein concentration was used by Bicinchoninic Acid P Protein Assay Kit (P0011, Beyotime, China). Sodium dodecyl sulfate-polyacrylamide gel (P0690, Beyotime, China) electrophoresis was used to separate each group of proteins (30 µg). Polyvinylidene fluoride membrane (HVLP04700, Millipore, USA) of appropriate size was cut according to the experimental needs, and the protein was transferred after the electrophoresis was completed. The membrane was then soaked with TBS, transferred to a box containing a skimmed milk powder solution, and sealed on a shaker at room temperature for at least one hour. Then the primary antibody diluted was added to the appropriate concentration with blocking solution to the sealed membrane and incubated for one hour at room temperature. The membranes were subsequently incubated with the secondary antibody under the same conditions, followed by chemiluminescent detection using the SignalFire ECL reagent (#6883, Cell Signaling Technology, USA) [27]. The protein bands were then quantified using ImageJ software. The primary and secondary antibodies were as follows: Nrf2 (1/500, ab313825, 110 kD), Bcl-2 (1/500, ab194583, 26 kD), HO-1 (1/2000, ab13243, 32 kD), cleaved Caspase-3 (1 µg/mL, ab184787, 17 kD), GAPDH (1/10000, ab181602, 36 kD), Goat Anti-Rabbit IgG H&L (HRP) (1:5000, ab205718), Marker [PR1910 (11-180 kD), PR1920 (11-245 kD), Solarbio, China].

Statistical Analysis

The data were presented as mean ± standard deviation (SD), and analyzed using SPSS statistical software (Version 21.0, Chicago, IL, USA). Two-group comparisons were analyzed using Student's *t* test and comparisons across more than two groups were conducted using one-way analysis of variance (ANOVA). Post hoc multiple comparisons were performed with Tukey's multiple comparisons [28]. $p < 0.05$ was considered as statistically significant.

Results

Effects of DARS2 Overexpression on Spatial Memory and Histopathological Damage in TLE Rats

In order to study the effect of *DARS2* on TLE model rats, the TLE model was successfully established and used. Compared with the control group, the expression of *DARS2* in hippocampal tissue of TLE model rats was apparently reduced, and the injection of *DARS2* overexpression plasmid could observably reverse the inhibition of *DARS2* expression by TLE model construction (Fig. 1A, $p < 0.05$). Moreover, compared with the control group, the average escape latency of TLE model rats was significantly longer (Fig. 1B, $p < 0.05$), and the dwell time and swimming distance of TLE model rats in the target quadrant were significantly reduced (Fig. 1C, $p < 0.05$). Interestingly, up-regulating the expression of *DARS2* could observably improve the performance of TLE rats in the water maze (Fig. 1B,C, $p < 0.05$).

HE staining revealed that the control group showed normal hippocampal tissue, intact cells, neat arrangement, clear layers, even staining, and clear nucleus (Fig. 1D). TLE group and TLE + NC group showed nerve cell degeneration, narrowing, loss, extensive edema, vacuolation, disordered neuron arrangement, poor laminarity, and uneven staining (Fig. 1D). However, compared with the TLE group, the TLE + *DARS2* group showed atrophy of hippocampal tissue cells, reduced cell loss, vacuolization in a few areas, disordered cell arrangement, recognizable cell layers, and most of the nuclei were visible, suggesting that up-regulation of *DARS2* could evidently improve the pathological damage of the hippocampus in TLE rats (Fig. 1D).

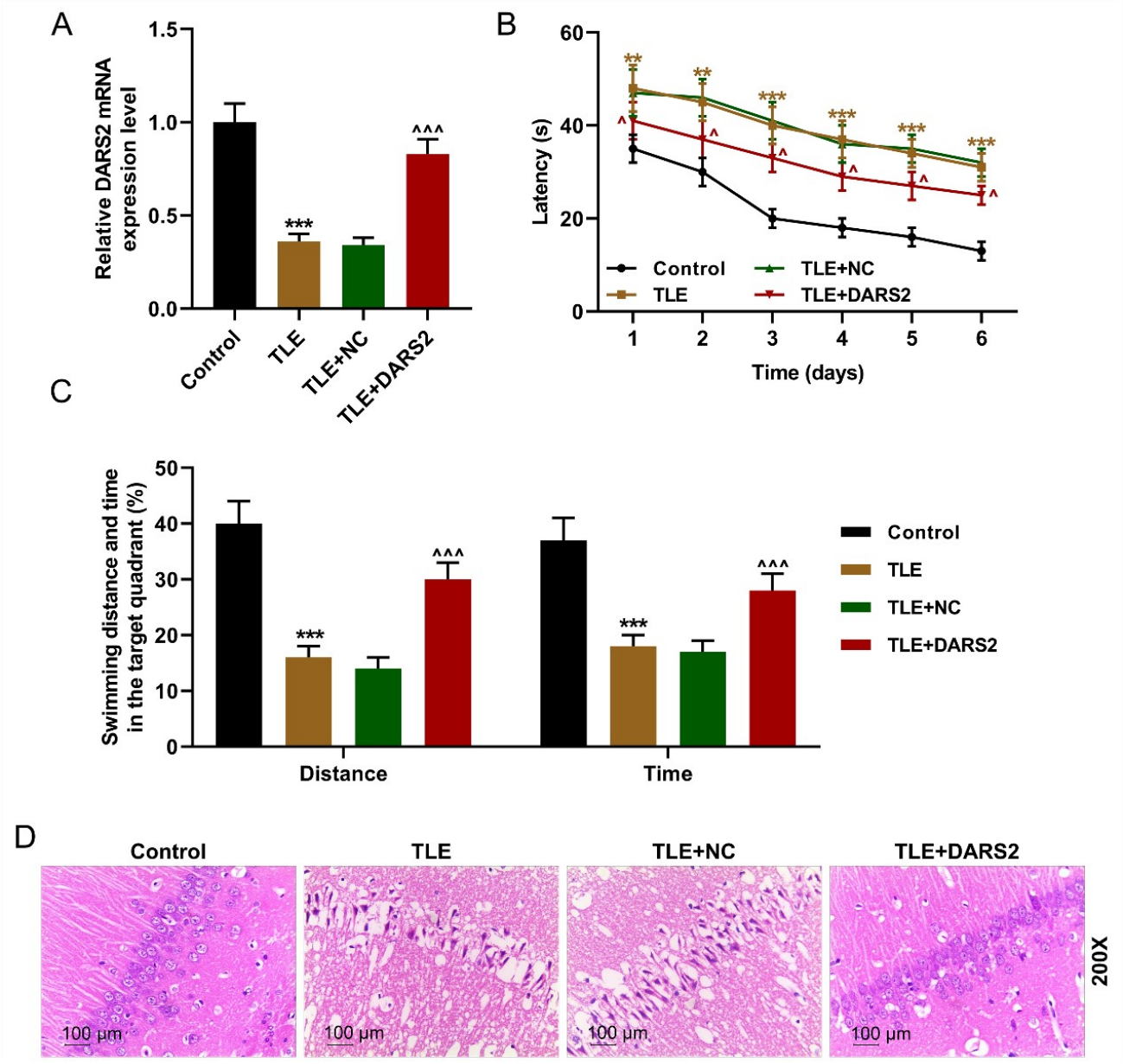

Fig. 1. Effects of aspartyl-tRNA synthetase 2 (*DARS2*) overexpression on spatial memory and histopathological damage in temporal lobe epilepsy (TLE) model rats. (A) *DARS2* expression of the control, TLE, TLE + negative control (NC), TLE + *DARS2* groups was detected by real-time quantitative PCR (RT-qPCR). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a control. After 4 weeks of interference treatment, the Morris water maze test was used to detect (B) average escape latency and (C) swimming time and distance in the target quadrant as a percentage of total time and distance. (D) After 4 weeks of interference treatment, the histopathological changes among the groups were detected by hematoxylin-eosin (HE) staining. Magnification, 200×, scale 100 μm. n = 10 each group. Results are expressed as the mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control; $^{\wedge}p < 0.05$, $^{\wedge\wedge\wedge}p < 0.001$ vs. TLE + NC, a one-way analysis of variance was used to compare multiple groups.

Effects of DARS2 Overexpression on Apoptosis, Inflammation and Antioxidation in Hippocampus of TLE Rats

Compared with the control group, apoptotic cells in the hippocampus of TLE rats were markedly increased, and the upregulation of *DARS2* significantly reduced the apoptosis of hippocampal tissue cells in TLE rats (Red fluorescence, Fig. 2A,B, $p < 0.05$). Besides, compared with the control group, the levels of inflammatory factors (TNF-α, IL-1β, IL-6) in the hippocampus of TLE rats were significantly increased (Fig. 2B–D, $p < 0.05$). But up-regulating *DARS2* can significantly reduce the levels of inflammatory factors in the hippocampus of TLE rats (Fig. 2C–E, $p < 0.05$). Furthermore, compared with the control group, the

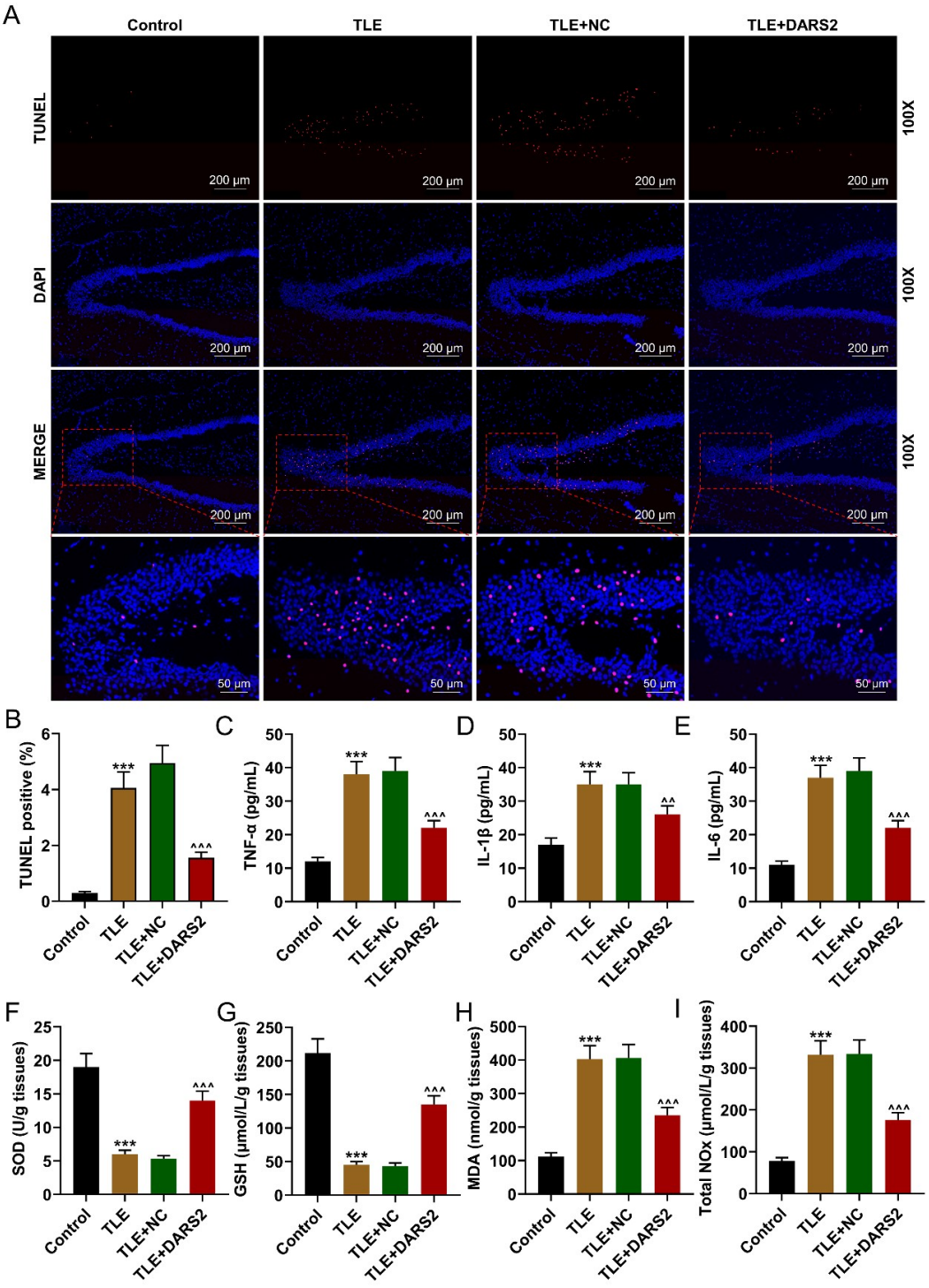

Fig. 2. Effects of *DARS2* overexpression on apoptosis, inflammation-related factors and antioxidant-related factors in the hippocampus of TLE rats. (A,B) Apoptosis rates of hippocampal tissue in the control, TLE, TLE + NC, TLE + *DARS2* groups were detected using the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Magnification, 100×, scale 200μm. (C–E) The contents of inflammatory factors Tumor necrosis factor-alpha (TNF-α), Interleukin-1 beta (IL-1β) and Interleukin-6 (IL-6) in hippocampus of each group were detected using ELISA. (F–I) The antioxidant enzymes superoxide dismutase (SOD) and glutathione (GSH), lipid peroxidation malondialdehyde (MDA), and total nicotinamideadenine dinucleotide phosphate oxidase (NADPH oxidase, Nox) content in hippocampal tissues of each group were detected using kits. n = 10 each group. Results are expressed as the mean ± standard deviation. *** $p < 0.001$ vs. Control; $^{\wedge\wedge}$ $p < 0.01$, $^{\wedge\wedge\wedge}$ $p < 0.001$ vs. TLE + NC, a one-way analysis of variance was used to compare multiple groups.

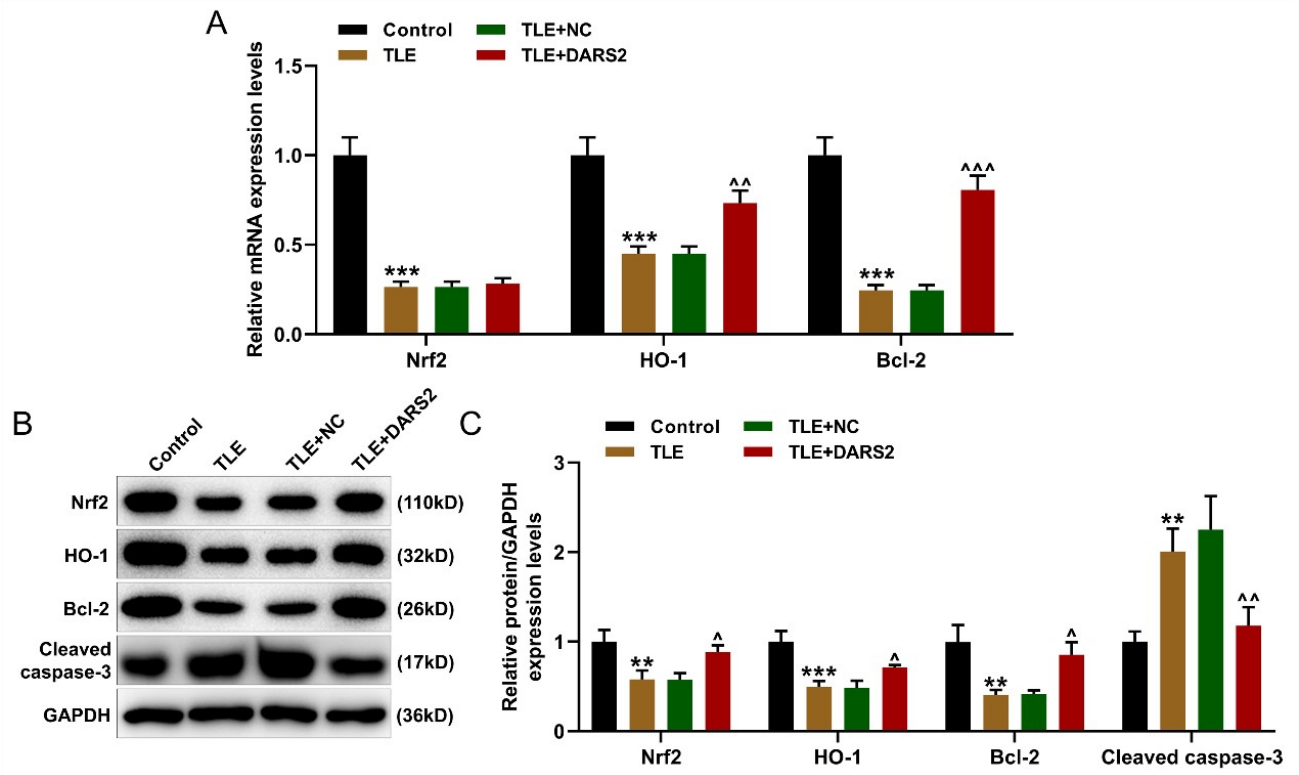

Fig. 3. Nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), B-cell lymphoma 2 (Bcl-2), and cleaved caspase-3 (protein) expression levels in the control, TLE, TLE + NC, and TLE + DARS2 groups in the hippocampus were detected by RT-qPCR (A) and Western blot (B,C) analyses. GAPDH served as a control. n = 10 each group. Results are expressed as the mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs. TLE + NC, a one-way analysis of variance was used to compare multiple groups.

antioxidant enzymes SOD and GSH in the hippocampus of TLE rats were evidently reduced, and the levels of MDA and total NOx were evidently increased (Fig. 2F–I, $p < 0.05$). Up-regulation of *DARS2* expression could observably increase the antioxidant capacity of hippocampal tissue in TLE rats, and can also reduce the levels of MDA and NOx in the tissue (Fig. 2F–I, $p < 0.05$).

In order to further explore the effect of *DARS2* on hippocampal tissue cell apoptotic activity and neural function recovery, RT-qPCR and Western blot analyses were used to detect Nrf2/HO-1 signaling pathway related proteins and apoptosis related proteins. The results showed that, compared with the control group, *Nrf2*, HO-1 and Bcl-2 in the hippocampus of TLE rats were observably reduced (Fig. 3A, $p < 0.05$). Upregulation of *DARS2* significantly increased the expression levels of HO-1 and Bcl-2 in the hippocampus of TLE rats ($p < 0.05$), but had no significant effect on the level of *Nrf2* mRNA (Fig. 3A). The results showed that, compared with the control group, TLE rats exhibited significantly decreased protein levels of Nrf2, HO-1, and Bcl-2 in the hippocampus, alongside a significant increase in cleaved caspase-3 (Fig. 3B,C, $p < 0.05$). Further studies revealed that upregulation of *DARS2* significantly elevated the protein expression levels of Nrf2, HO-1, and Bcl-2, and reduced the protein level of cleaved caspase-3 in the hippocampus of TLE rats (Fig. 3B,C, $p < 0.05$).

Nrf2 Silencing Partially Reversed the Effect of DARS2 on Spatial Memory and Histopathological Damage in TLE Rats

To further verify the role of *DARS2* and *Nrf2* in TLE, a TLE rat model was used in subsequent experiments. Results as expected, at the mRNA level, the expression of *DARS2* in the TLE + shNrf2 + DARS2 group was not significantly different from TLE + shNC + DARS2 group (Fig. 4A). However, at the protein level, DARS2 significantly increased the protein level of Nrf2, and shNrf2 significantly reversed the effect of DARS2 overexpression on the Nrf2 protein level (Fig. 4B,C, $p < 0.05$). Moreover, *DARS2* overexpression could markedly shorten the escape latency of TLE rats, and shNrf2 significantly inhibited the improvement of *DARS2* overexpression on the performance of the water maze test in TLE rats (Fig. 4D,E, $p < 0.05$). The HE staining experiment showed that the hippocampal tissue of the TLE group was seriously damaged, and the TLE + DARS2 group could partially improve the degree of tissue damage (Fig. 4F). More importantly, in the TLE + shNrf2 + DARS2 group, the degeneration of nerve cells was

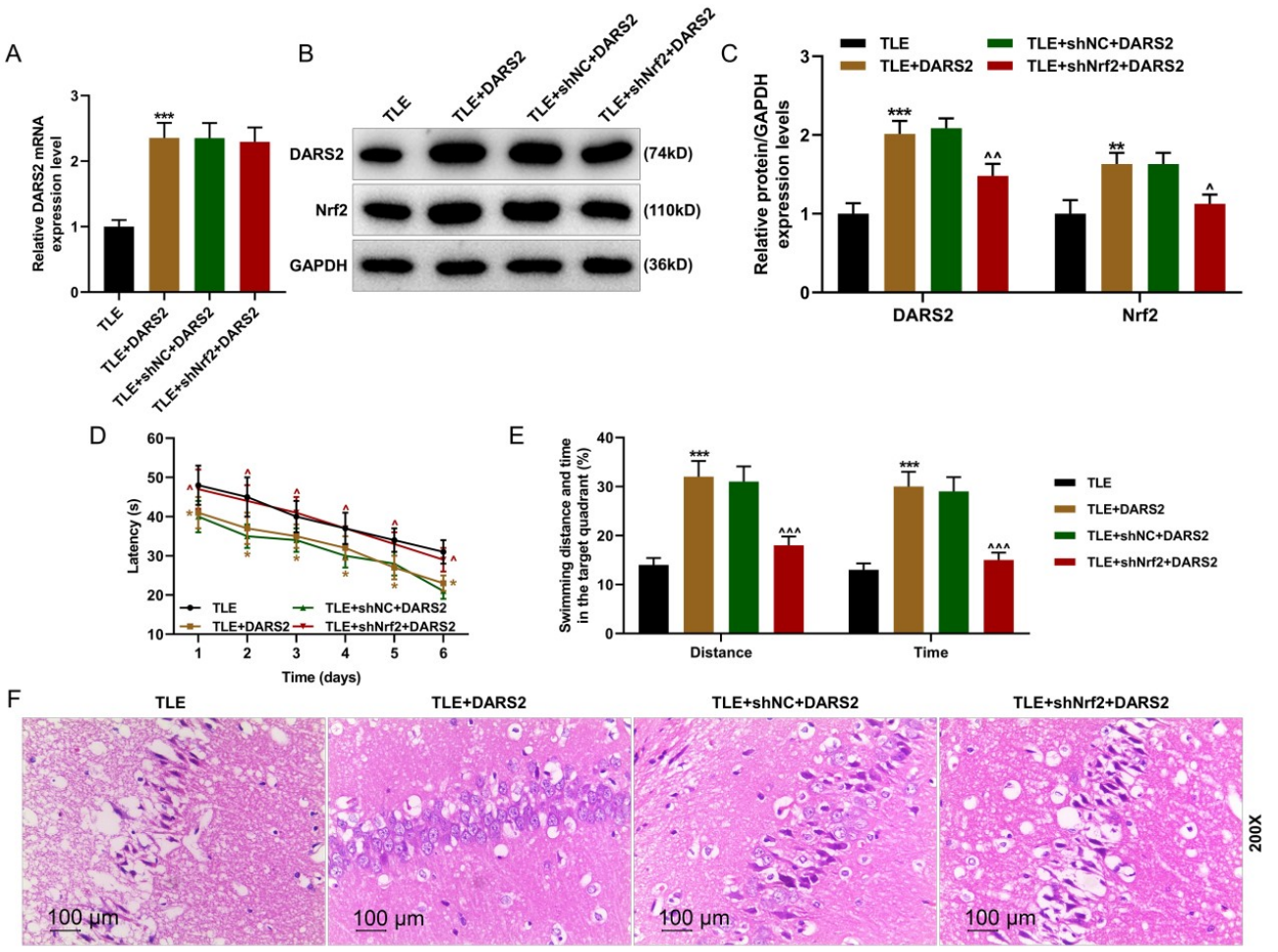

Fig. 4. *Nrf2* silencing partially reversed the effect of *DARS2* overexpression on spatial memory and histopathological damage in TLE rats. (A) RT-qPCR was used to detect *DARS2* mRNA levels in TLE, TLE + DARS2, TLE + DARS2 + shNC, TLE + DARS2 + shNrf2 groups. (B,C) Nrf2 protein levels in each group were detected by Western blot analysis. GAPDH served as a control. The Morris water maze test was used to detect (D) mean escape latency and (E) swimming time and distance in the target quadrant as a percentage of total time and distance. (F) The histopathological changes among the groups were detected using HE staining. Magnification, 200×, scale 100 μm. n = 10 each group. Results are expressed as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TLE; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs. TLE + shNC + DARS2, a one-way analysis of variance was used to compare multiple groups.

serious, the loss of cells was increased, the vacuolation was serious, the arrangement of nerve cells was disordered, the layering was poor, and the staining was uneven, suggesting that *Nrf2* silencing could reverse the ameliorative effect of *DARS2* overexpression on hippocampal injury in TLE rats (Fig. 4F).

Nrf2 Silencing Partially Reversed the Effect of DARS2 on Apoptosis, Inflammation and Antioxidation in the Hippocampus of TLE Rats

The effects of *DARS2* and *Nrf2* on pathophysiological characteristics and apoptosis of TLE rats require further investigation. The results showed that *Nrf2* silencing apparently reduced the effect of *DARS2* overexpression on the inhibition of hippocampal tissue cell apoptosis in TLE rats (Red fluorescence, Fig. 5A,B, $p < 0.05$). The levels of TNF-α, IL-1β, and IL-6 in the TLE + DARS2 group were apparently lower than those in the TLE group, while the levels of inflammatory factors in the TLE + shNrf2 + DARS2 group were markedly increased compared with the TLE + shNC + DARS2 group (Fig. 5C–E, $p < 0.05$). *Nrf2* silencing apparently inhibited the effect of DARS2 overexpression on the antioxidant capacity of hippocampus in TLE rats (Fig. 5F–I, $p < 0.05$). Compared with the TLE group, the expression levels of HO-1 and Bcl-2 were significantly increased, while the expression of caspase-3 was significantly decreased in the TLE + *DARS2* group (Fig. 6A, $p < 0.05$). However, *Nrf2* silencing obviously reversed the upregulatory effect of *DARS2* overexpression on HO-1 and Bcl-2 expression, as well as the inhibitory effect on

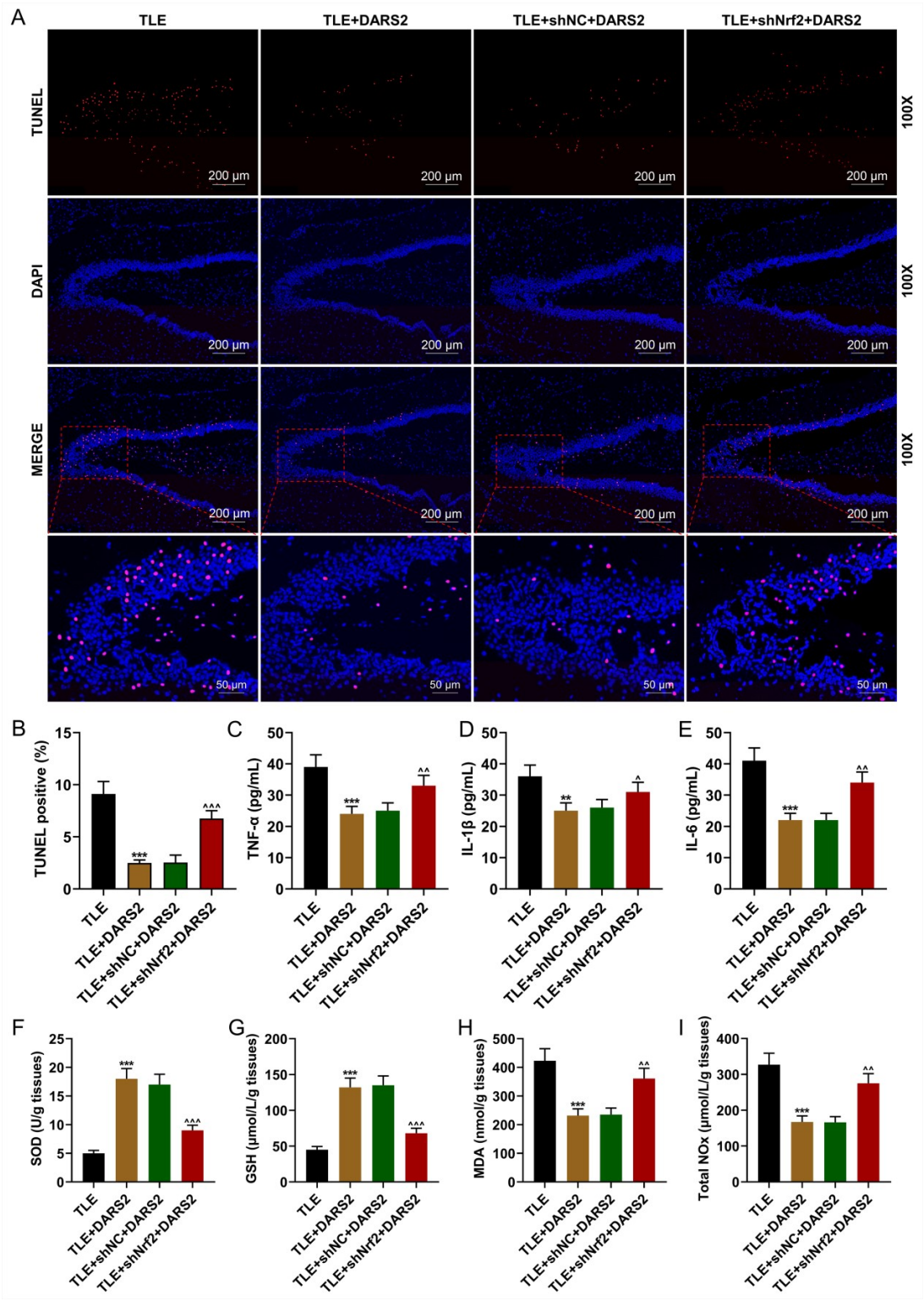

Fig. 5. *Nrf2* silencing partially reversed the effects of *DARS2* on apoptotic cells, inflammation-related factors, and antioxidant-related factors in the hippocampus of TLE rats. (A,B) Apoptosis rates of hippocampal tissue was detected using TUNEL assay in the hippocampus of TLE, TLE + DARS2, TLE + DARS2 + shNC, TLE + DARS2 + shNrf2 groups. Magnification, 100×, scale 200 μm. (C–E) The contents of inflammatory factors (TNF-α, IL-1β and IL-6) in the hippocampus of each group were detected using ELISA. (F–I) SOD and GSH, MDA, and total NOx content in the hippocampus of each group were detected using kits. n = 10 each group. Results are expressed as the mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TLE; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs. TLE + shNC + DARS2, a one-way analysis of variance was used to compare multiple groups.

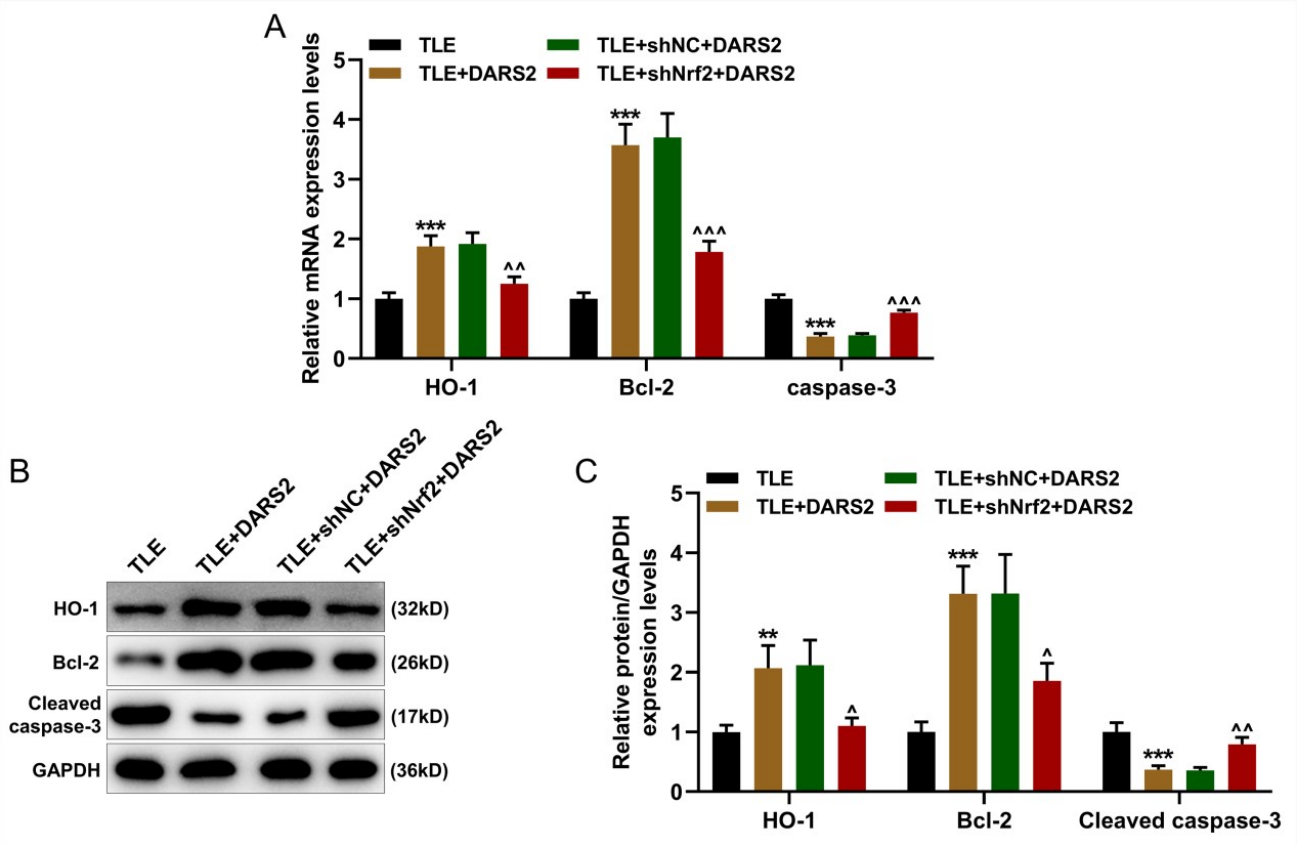

Fig. 6. The expression levels of HO-1, Bcl-2 and cleaved caspase-3 (protein) of the TLE, TLE + DARS2, TLE + DARS2 + shNC, TLE + DARS2 + shNrf2 groups were detected by RT-qPCR (A) and Western blot (B,C) analyses. GAPDH served as a control. Results are expressed as the mean ± SD. n = 10 each group. Results are expressed as the mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TLE; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs. TLE + shNC + DARS2, a one-way analysis of variance was used to compare multiple groups.

caspase-3 levels, in the hippocampus of TLE rats (Fig. 6A, $p < 0.05$). In addition, the expression of HO-1 and Bcl-2 increased significantly, and cleaved caspase-3 decreased significantly in the TLE + DARS2 group compared with the TLE group (Fig. 6B,C, $p < 0.05$); whereas *Nrf2* silencing apparently reversed the effect of *DARS2* overexpression on the expression of HO-1 and Bcl-2 in the hippocampus of TLE rats and the inhibitory effect of cleaved caspase-3 protein levels (Fig. 6B,C, $p < 0.05$).

Discussion

At present, the diagnosis of epilepsy is mainly based on clinical symptoms, neuroimaging and EEG [29,30]. Emerging evidence suggests that molecular biomarkers may provide additional insights into disease mechanisms and prognosis. The prognostic biomarker *DARS2* has been shown to be associated with immune infiltration in bladder tumors [31]. Moreover, *DARS2* expression is negatively correlated with prognosis in lung adenocarcinoma (LUAD) and can effectively predict patient outcomes [32]. We investigated the expression levels of *DARS2* and *Nrf2* in hippocampal tissues of TLE rats, and explored their effects on the pathophysiological characteristics and apoptosis of TLE rats and their regulatory relationships. We found that *DARS2* was significantly lower expressed in hippocampal tissue of TLE rats, and up-regulation of *DARS2* can effectively reduce hippocampal tissue cell apoptosis, reduce inflammatory factor levels, increase antioxidative capacity in rats, and improve spatial memory learning ability and hippocampal histopathological damage in rats. These findings indicate that *DARS2* plays a critical role in TLE and holds potential as a valuable biomarker.

Research has shown that seizures are closely related to oxidative stress [8]. On the one hand, seizures can cause mitochondrial damage, and on the other hand, mitochondrial dysfunction can cause seizures [9,33]. *Nrf2* is a key transcription factor that regulates the antioxidant response in the body, and under normal physiological conditions, *Nrf2* is located in the cytoplasm to maintain its low physiological state [34]. When the body is attacked by oxygen-free radicals and endogenous toxins, *Nrf2* translocates into the nucleus and combines with the antioxidant response element ARE to induce the expression level of antioxidant pro-

teins after transcription [35]. Heme oxygenase-1 (HO-1) is a critical antioxidant enzyme in the *Nrf2* pathway [36]. Research has reported that upregulating HO-1 expression in the central nervous system can reduce the toxic effects of oxygen-free radicals and endogenous toxins on neurons [37]. Further exploration using a TLE rat model revealed that *Nrf2* silencing partially reversed the effects of *DARS2* on spatial memory, histopathological damage, inflammation, apoptosis, and antioxidation in TLE rats. It is suggested that *DARS2* activates *Nrf2* pathway to protect the brain from seizure damage. Compared with previous studies, our study shows that *DARS2* has more potential advantages in treating epilepsy, and further enriches the network regulation mechanism of *Nrf2* pathway in epilepsy.

This study has several limitations. First, the control group received only an intraperitoneal injection of normal saline, without undergoing hippocampal stereotactic puncture. The setting of the control group was not rigorous enough, which may affect the results and lead to deviations in surgical outcomes. Second, the findings are based on a rat model of temporal lobe epilepsy, which may not fully capture the complexity of human TLE. Moreover, although *Nrf2* was identified as a downstream mediator, the precise mechanisms by which *DARS2* regulates the *Nrf2* pathway remain incompletely understood. Future studies will aim to elucidate the detailed regulatory mechanisms of *DARS2* on *Nrf2* and validate these findings in clinical samples.

Conclusion

In summary, this study confirms that *DARS2* expression is downregulated in TLE rat models, and that it may improve brain damage resulting from epilepsy by activating the *Nrf2* signaling pathway, providing meaningful theoretical evidence for the future treatment of epilepsy.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author Contributions

JD and JH designed the research study. HW and PD performed the research. JL and PW collected and analyzed the data. JD has been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity are addressed.

Ethics Approval and Consent to Participate

All animals were used in compliance with the Regulations on the Administration of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (MOST version), and approved by the Zhejiang Provincial Experimental Animal Center Animal Welfare and Ethics Committee [No. ZJCLA-IACUC-20011150].

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Conflict of Interest

The authors declare no conflict of interest.

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