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ORIGINAL RESEARCH

Molecular mechanism research of the crude extract of Angong Niuhuang Pill inhibiting GPX4-mediated ferroptosis in mice with juvenile sepsis associated encephalopathy

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Abstract

Background: Sepsis associated encephalopathy (SAE) is a prevalent form of organ dysfunction associated with sepsis. There is no overt central nervous system (CNS) infection accompanying it, yet it carries a significant risk of mortality and can lead to long-lasting neurological complications. The efficacy of Angong Niuhuang Pill (AGNH) in enhancing conditions like cerebral ischemia, cerebral trauma and sepsis has been well-established. Nonetheless, the specific regulatory roles and underlying mechanisms of AGNH in the progression of SAE remain unexplored. **Methods**: The lipopolysaccharide (LPS) treatment was utilized to construct SAE rat model. Berderson's neurological examination scoring system was used for scoring. The levels of genes and iron content were examined through enzyme-linked immunosorbent assay (ELISA) or the corresponding commercial kits. The prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB) level were confirmed through the automatic coagulation analyzer. The number and morphology of neurons were evaluated through hematoxylin eosin (HE) staining. The protein expressions were determined through western blot. **Results**: The increased Berderson never function score mediated by LPS treatment was attenuated after AGNH or Defetoxamine (DFO, ferroptosis inhibitor) treatment, indicating that AGNH improved neurobehavioral function in juvenile SAE mice. Furthermore, AGNH improved inflammation and coagulation parameters in young SAE mice. AGNH promoted neuronal growth and mitigated neuronal damage in juvenile SAE mice. Additionally, AGNH inhibited ferroptosis and reduced oxidative stress in young SAE mice. Lastly, it was demonstrated that AGNH promoted nuclear factor erythroid 2-related factor 2 (Nrf2)/glutathione peroxidase 4 (GPX4) signaling pathway through up-regulating the Nrf2 and GPX4 protein expressions. **Conclusions**: This study revealed a novel finding that AGNH has the ability to inhibit GPX4-induced ferroptosis in juvenile SAE mice by modulating the Nrf2/GPX4 signaling pathway. This breakthrough implies that AGNH has promising prospects as a therapeutic agent for SAE.

Keywords

AGNH; Ferroptosis; SAE; Nrf2/GPX4 pathway

1. Introduction

Sepsis is one of the global health problems that badly threaten the lives of human [1, 2]. In cases of sepsis, the dysregulated inflammatory response can lead to a variety of organ dysfunctions [3]. In the early stage of sepsis, the central nervous system (CNS) is influenced, and then burgeoned into sepsis-associated encephalopathy (SAE), accounting for about 50% of sepsis patients [4, 5]. SAE is characterized by widespread brain dysfunction, including hallucinations, changes in consciousness, lack of attention and delirium [6]. Nevertheless, the existing treatment options for SAE are constrained, and given its significant morbidity and mortality rates, there is a pressing necessity to investigate the pathophysiology of SAE and search for innovative pharmaceuticals for clinical intervention.

Angong Niuhuang Pill (AGNH), firstly published in the book "Wen Bing Tiao Bian" in the Qing dynasty, is a widely recognized traditional Chinese patent medicine used to treat various cerebrovascular diseases [7, 8]. AGNH is mainly composed of 11 Chinese herbs, such as Bovis Calculus,

Rhinoceros Horn, Moschus, Margarita, Cinnabaris, Realgar, Coptidis Rhizoma, Scutellariae Radix, Pearl, Gardeniae Fructus, Curcumae Radix and Borneolum Syntheticum. Flavonoids, iridoid glycosides, and alkaloids are the primary bioactive compounds found in AGNH. Research has shown that AGNH can effectively ameliorate certain ailments. One notable example is its ability to regulate the T helper cell 17 (Th17)/Regulatory T cells (Treg) immune equilibrium and alleviate persistent inflammation in atherosclerosis in apolipoprotein E (ApoE)-/- mice models [9]. In addition, AGNH retards the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (PIK3CG)/p65/matrix metalloproteinase 9 (MMP9) signaling to against LPS-triggered acute lung injury [10]. Moreover, AGNH demonstrates positive effects in the treatment of chronic obstructive pulmonary disease [11]. Importantly, AGNH has also participated into the regulation of the brain-related diseases. For instance, AGNH can address gut microbiota dysbiosis to alleviate cerebral ischemia/reperfusion injury [12]. Furthermore, AGNH reduces ferroptosis in ischemic and hemorrhagic stroke [13]. Additionally, AGNH possesses neuroprotective properties that enhance brain injury caused by ischemia/reperfusion [14]. However, the regulatory functions and related mechanism of AGNH in SAE progression keep vague, and need further investigated.

In this work, it is focused on studying the relationship between AGNH and SAE progression on regulating glutathione peroxidase 4 (GPX4)-mediated ferroptosis. The research findings revealed that AGNH has the potential to inhibit GPX4-induced ferroptosis in juvenile SAE mice by modulating the Nrf2/GPX4 signaling pathway. This study suggests that AGNH could serve as a promising candidate for the pharmaceutical management of SAE.

2. Materials and methods

2.1 AGNH preparation

AGNH was purchased from Guangzhou Baiyunshan Zhongyi pharmaceutical co., Ltd (Z44020047, Guangzhou, Guangdong, China). For preparation, AGNH was ground and dissolved in normal saline using a mortar to form suspensions of 100 mg/mL.

2.2 Mice model

All experiments were executed by the Guide for the Care and Use of Laboratory Animals (Ministry of Health, China), and the approval of this work was gained from the Committee of First Affiliated Hospital of Xinjiang Medical University (Approval No. 20230901-11). All mice were kept with free food and water, with humidity about 65%, temperature about 25 °C and a 12 h light/dark cycle.

Twenty-four male C57BL/6 mice (8–10 week) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were randomly divided into sham group, lipopolysaccharide (LPS) group, LPS + AGNH group and LPS + DFO group (N = 6 mice in each group). The sham group was subjected to no treatment. LPS (Escherichia coli endotoxin 055:B5, L2880, Sigma-Aldrich, St. Louis, MO, USA) was administered at 5 mg/kg by intraperitoneal injection to establish the SAE mouse model [15, 16]. The mice in the AGNH or DFO treatment group were administered AGNH or DFO at a dosage of 100 mg/kg through intraperitoneal injection one hour post-LPS treatment. This dosage was calculated based on the mice's body weight to mirror clinical drug administration. Behavioral assessments were conducted after a 24-hour period. Subsequently, the mice were anesthetized using 2% isoflurane inhalation, euthanized by decapitation and their brain tissue was excised and promptly frozen.

2.3 Berderson's neurological examination scoring system

The mice's behaviors were scored through Berderson's neurological examination scoring system. Scores "0": no apparent deficit (normal); "1": not fully extend their left front paw (mild deficit), "2": circled to the contralateral side (moderate deficit); "3": losing the righting reflexes and the ability (severe deficit). After 24 h induction, neurological deficit scores were carefully evaluated in each mouse by an observer blinded to the study.

2.4 ELISA

The inflammatory cytokines were determined through ELISA. The tumor necrosis factor-alpha (TNF-α) ELISA kit (ab208348, Abcam, Shanghai, China) and interleukin-6 (IL-6) ELISA kit (ab222503, Abcam, Shanghai, China) were employed to examine the levels of TNF-α and IL-6 in serum in line with the corresponding ELISA kits.

2.5 Detection of PT, APTT, TT and FIB

The coagulation function was assessed by analyzing parameters including prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB) level using the Sysmex CS-5100 automatic coagulation analyzer (Sysmex Medical Electronics Co., Kobe, Japan).

2.6 Hematoxylin eosin (HE) staining

After being fixed with 4% paraformaldehyde, the brains of mice were embedded in paraffin. Brain tissues (Hippocampus and Cortex) were cut into the 4 μm thickness sections for HE staining. The sections were subjected to xylene for 5 min, 100% ethanol for 10 min, 95% ethanol for 5 min and 75% ethanol for 5 min. After washing, the sections were mixed with hematoxylin and eosin for staining. The images were viewed with the Leica inverted optical microscope (DMi1, Leica, Mannheim, BW, Germany).

2.7 Detection of iron content, MDA, GSH and SOD

The obtained hippocampal tissues were homogenized, and then evaluated for hippocampal iron content through the iron assay kit (ab83366, Abcam, Shanghai, China).

The levels of malondialdehyde (MDA, ab118970, Abcam, Shanghai, China), glutathione (GSH, ab65322, Abcam, Shanghai, China) and superoxide dismutase (SOD, ab65354, Abcam,

Shanghai, China) in brain tissues were measured through the commercial kits.

2.8 Western blot

Total proteins from hippocampal tissues were isolated through the radio immunoprecipitation assay (RIPA) lysis buffer, then separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), next moving to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Post sealing with non-fat milk, the append of primary antibodies were made in the membranes for 12 h, next for secondary antibody (1:2000, ab288151, Abcam, Shanghai, China) for another 2 h. Finally, the protein blots were assessed through the chemiluminescence detection kit (20148, Thermo Fisher Scientific, Waltham, MA, USA).

The primary antibodies: Nrf2 (1/1000, ab62352, Abcam, Shanghai, China), GPX4 (1/1000, ab125066, Abcam, Shanghai, China) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1/500, ab8245, Abcam, Shanghai, China).

2.9 Statistical analysis

The data were presented as mean ± standard deviations (SD). The data analysis was conducted through SPSS 20.0 (IBM Corp., Armonk, NY, USA). Comparisons in multiple groups were performed by one-way Analysis of Variance (ANOVA) followed by the Turkey test. $p < 0.05$ was deemed as statistically significant.

3. Results

3.1 AGNH improved neurobehavioral function in juvenile SAE mice

The SAE mouse model was established with 5 mg/kg LPS by intraperitoneal injection. After 24 h, the mice's behaviors were assessed through Berderson's neurological examination scoring system. Following the administration of LPS, there was a notable elevation in the Berderson nerve function score, rising from 0 to 2.3 ($p < 0.001$). However, this elevation was counteracted by subsequent treatments with AGNH, resulting in a reduction from 2.3 to 1 ($p < 0.001$), as well as with Defetoxamine (DFO), a ferroptosis inhibitor, leading to a decrease from 2.3 to 0.8 ($p < 0.001$) (Fig. 1), suggesting that AGNH improved neurobehavioral function in juvenile SAE mice.

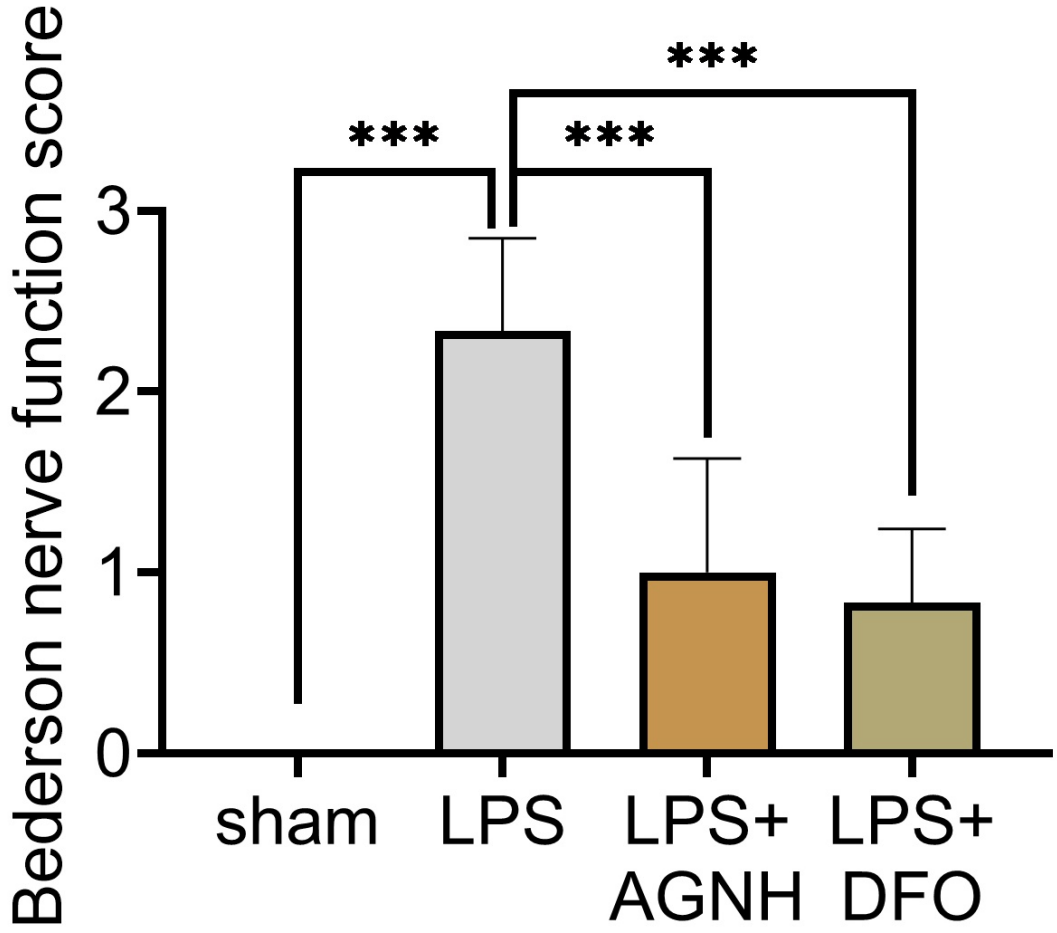

FIGURE 1. AGNH improved neurobehavioral function in juvenile SAE mice. Mice were separated into the sham, LPS, LPS + 100 mg/kg AGNH and LPS + 100 mg/kg DFO groups. Berderson's neurological examination scoring system was used for scoring. N = 6 mice in each group. ***$p < 0.001$. LPS: lipopolysaccharide; AGNH: Angong Niuhuang Pill; DFO: Defetoxamine.

3.2 AGNH ameliorated inflammation and coagulation function in juvenile SAE mice

Next, the regulatory impacts of AGNH on inflammatory cytokines were determined. Results from ELISA uncovered that the pro-inflammatory factors TNF-α and IL-6 levels were both enhanced after LPS treatment, but these effects were attenuated after AGNH or DFO treatment (Fig. 2A). The prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) were increased, and fibrinogen (FIB) level were examined for evaluating coagulation function. The findings indicated that levels of PT, APTT and TT showed an increment, while FIB levels exhibited a decrease following LPS administration. However, these alterations were reversed upon treatment with either AGNH or DFO as illustrated in Fig. 2B. These results suggest that AGNH helped alleviate inflammation and improve coagulation function in juvenile SAE mice.

3.3 AGNH alleviated neuronal injury in juvenile SAE mice

Through HE staining, the number and morphology of neurons (Hippocampus and Cortex) were evaluated. The number of neurons (Hippocampus and Cortex) was reduced after LPS treatment, but this change was offset after AGNH or DFO treatment (Fig. 3, $p < 0.001$), indicating AGNH alleviated neuronal injury in juvenile SAE mice.

3.4 AGNH suppressed ferroptosis and oxidative stress in juvenile SAE mice

The regulatory functions of AGNH on ferroptosis and oxidative stress were examined. The iron content was elevated after LPS treatment, but this change was counteracted after AGNH or DFO treatment (Fig. 4A). Moreover, following LPS treatment, there was an elevation in MDA levels alongside a reduction in GSH and SOD levels, with these impacts being reversed by AGNH or DFO treatment (Fig. 4B). Collectively, AGNH effectively inhibited ferroptosis and mitigated oxidative stress in young SAE mice.

3.5 AGNH promoted Nrf2/GPX4 signaling pathway

The Nrf2/GPX4 pathway is involved in various inflammatory diseases, and then the regulatory effects of AGNH on the Nrf2/GPX4 signaling in SAE progression were further investigated. Following LPS treatment, the protein levels of Nrf2 and GPX4 exhibited a decrease as observed through western blot analysis. However, the administration of AGNH or DFO led to a restoration of these alterations (Fig. 5). In short, AGNH promoted Nrf2/GPX4 signaling pathway.

4. Discussion

The traditional Chinese medicines have been reported to own important regulatory functions in the treatment of SAE [17–19]. AGNH has been discovered to participate into the regulation of some diseases [9–14]. However, the regulatory effects of AGNH and related mechanism in the progression of SAE keep indistinct. The study demonstrated that the elevated Berderson motor function score induced by LPS treatment was mitigated following AGNH or DFO treatment, suggesting that AGNH enhanced neurological function in young SAE mice. Moreover, AGNH improved the inflammatory response in juvenile SAE mice by decreasing levels of TNF-α and IL-6, while also enhancing coagulation function through increased PT, APTT and TT and reduced FIB level. Additionally, AGNH boosted neuronal activity and mitigated neuronal damage in juvenile SAE mice.

Ferroptosis is one iron-dependent programmed cell death that differs from cell apoptosis, necrosis and autophagy [20–22]. Currently, there is a wealth of evidence suggesting that ferroptosis plays a crucial role in the progression of SAE. Therefore, targeting ferroptosis inhibition may represent a novel therapeutic avenue. One illustration of this concept is the influence of exosome-mediated lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) on the miR-9-5p/transferrin receptor (TFRC) and glutamate oxaloacetate transaminase 1 (GOT1) axis, which enhances ferroptosis and consequently exacerbates the advancement of SAE [23]. Acetaminophen regulates the GPX4 pathway to relieve ferroptosis in SAE mice [24]. Furthermore, autophagy degrades transferrin receptor 1 (TFR1) to inhibit ferroptosis, thereby relieving cognitive dysfunction in SAE mice [25]. In this work, it was confirmed that AGNH suppressed ferroptosis and oxidative stress in juvenile SAE mice.

Nuclear factor erythroid 2-related factor 2 (Nrf2) acts as a transcription factor that combats oxidative stress by activating a wide range of genes involved in lipid peroxidation and iron metabolism. Consequently, it plays a crucial role in inhibiting ferroptosis [26]. The nuclear Nrf2 can combine with antioxidant response element (ARE) to trigger GPX4 expression, thereby maintaining redox homeostasis and modulating ferroptosis [27, 28]. This Nrf2/GPX4 signaling pathway has been proved to participate into the regulation of ferroptosis in various diseases. For example, protein arginine methyltransferase 4 (PRMT4) retards the Nrf2/GPX4 pathway to heighten ferroptosis, thereby accelerating doxorubicin-triggered cardiomyopathy [29]. Moreover, zinc plays a role in activating the Nrf2/GPX4 defense pathway to mitigate ferroptosis and promote functional recovery in contusion spinal cord injury [30]. Besides, protein deglycase DJ-1 (Parkinson disease protein 7) stimulates the Nrf2/GPX4 signal pathway to attenuate trophoblast ferroptosis in preeclampsia [31]. It is crucial to note that the activation of the Nrf2/GPX4 pathway by irisin plays a significant role in mitigating ferroptosis in SAE [32]. However, the regulatory effects of AGNH on the Nrf2/GPX4 signaling pathway in SAE progression keep vague. Lastly, this study showed that AGNH enhanced the Nrf2/GPX4 signaling pathway by increasing the levels of Nrf2 and GPX4 proteins.

5. Conclusions

In conclusion, this work for the first time manifested that AGNH can suppress GPX4-mediated ferroptosis in mice with juvenile SAE through regulating Nrf2/GPX4 signaling pathway. Nevertheless, there are some limitations in this study,

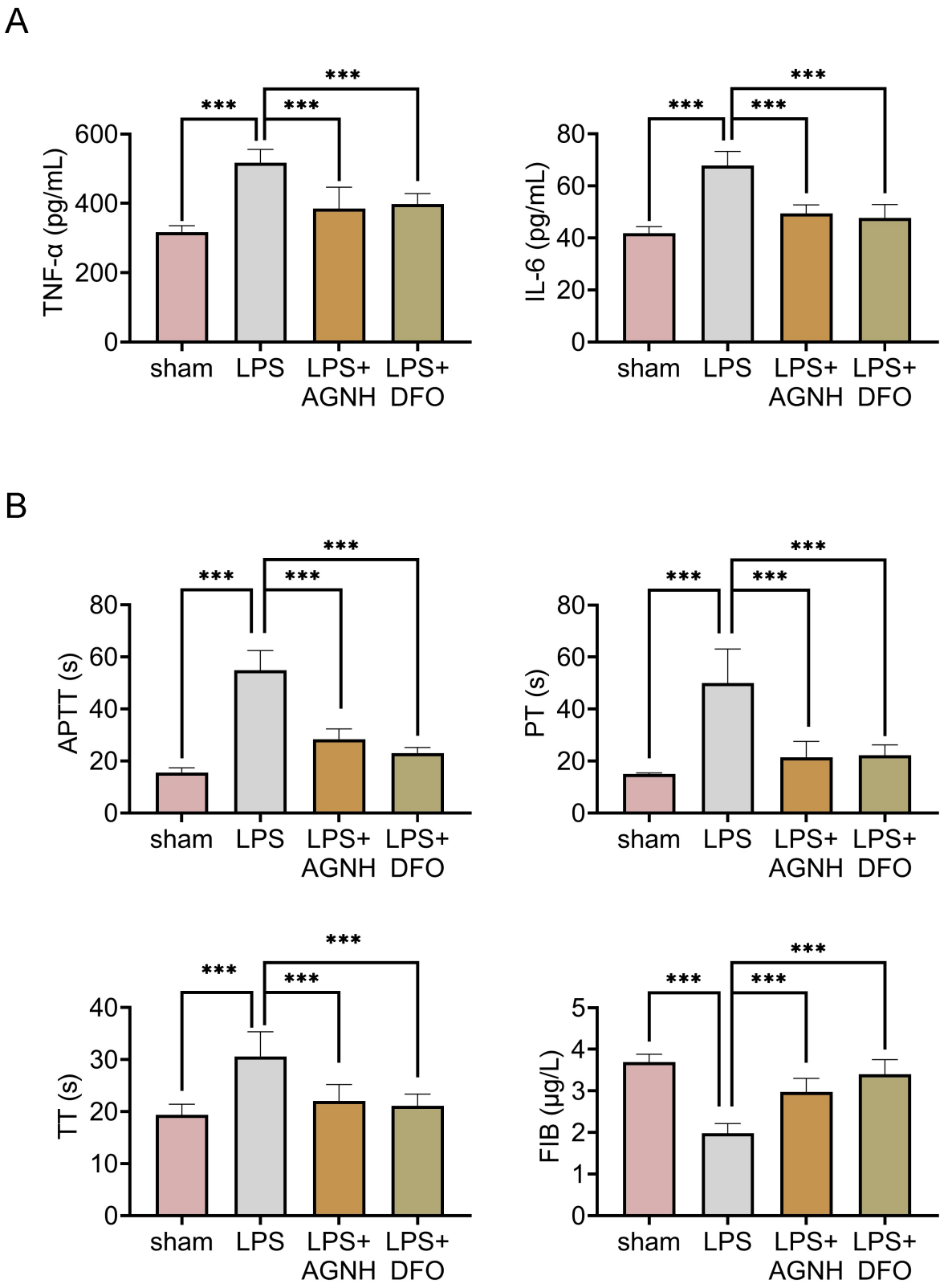

FIGURE 2. AGNH ameliorated inflammation and coagulation function in juvenile SAE mice. Mice were separated into the sham, LPS, LPS + 100 mg/kg AGNH and LPS + 100 mg/kg DFO groups. (A) The levels of TNF-α and IL-6 were examined through ELISA. (B) The prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB) level were confirmed through the automatic coagulation analyzer. N = 6 mice in each group. ***$p <$ 0.001. LPS: lipopolysaccharide; AGNH: Angong Niuhuang Pill; DFO: Defetoxamine; TNF-α: tumor necrosis factor-alpha; IL-6: interleukin-6.

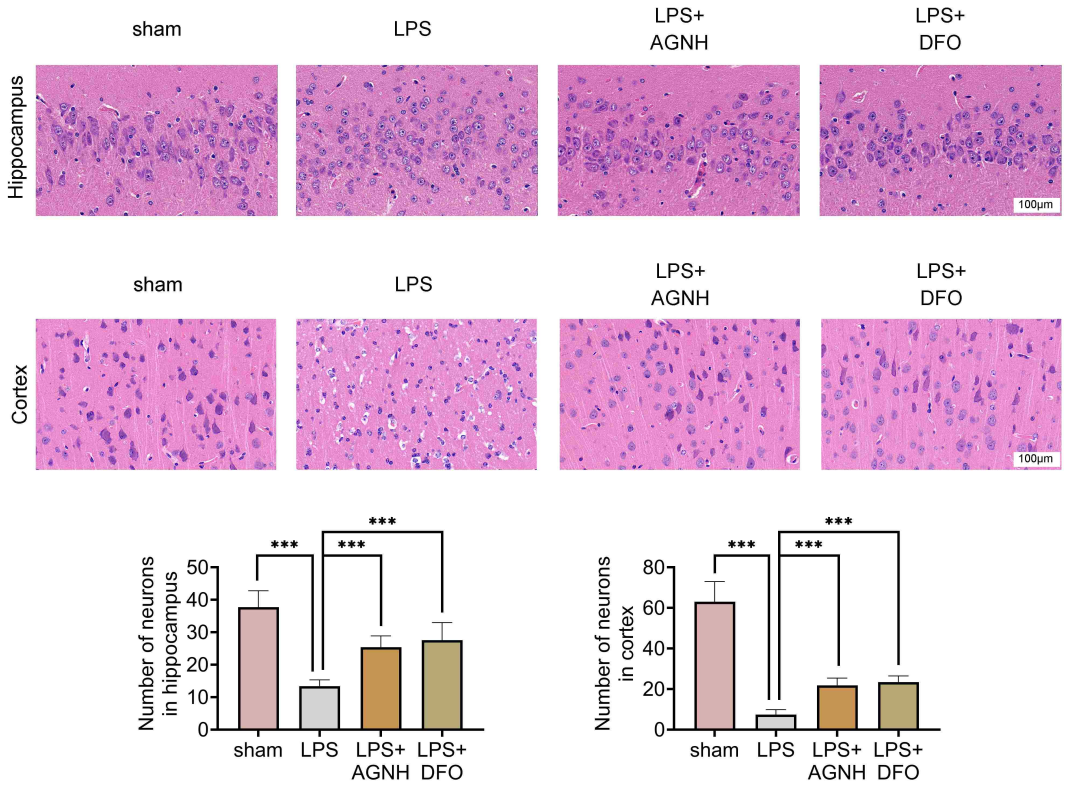

FIGURE 3. AGNH alleviated neuronal injury in juvenile SAE mice. Mice were separated into the sham, LPS, LPS + 100 mg/kg AGNH and LPS + 100 mg/kg DFO groups. The number and morphology of neurons (Hippocampus and Cortex) were evaluated through HE staining. Bar = 100 μm. N = 6 mice in each group. ***$p < 0.001$. LPS: lipopolysaccharide; AGNH: Angong Niuhuang Pill; DFO: Defetoxamine.

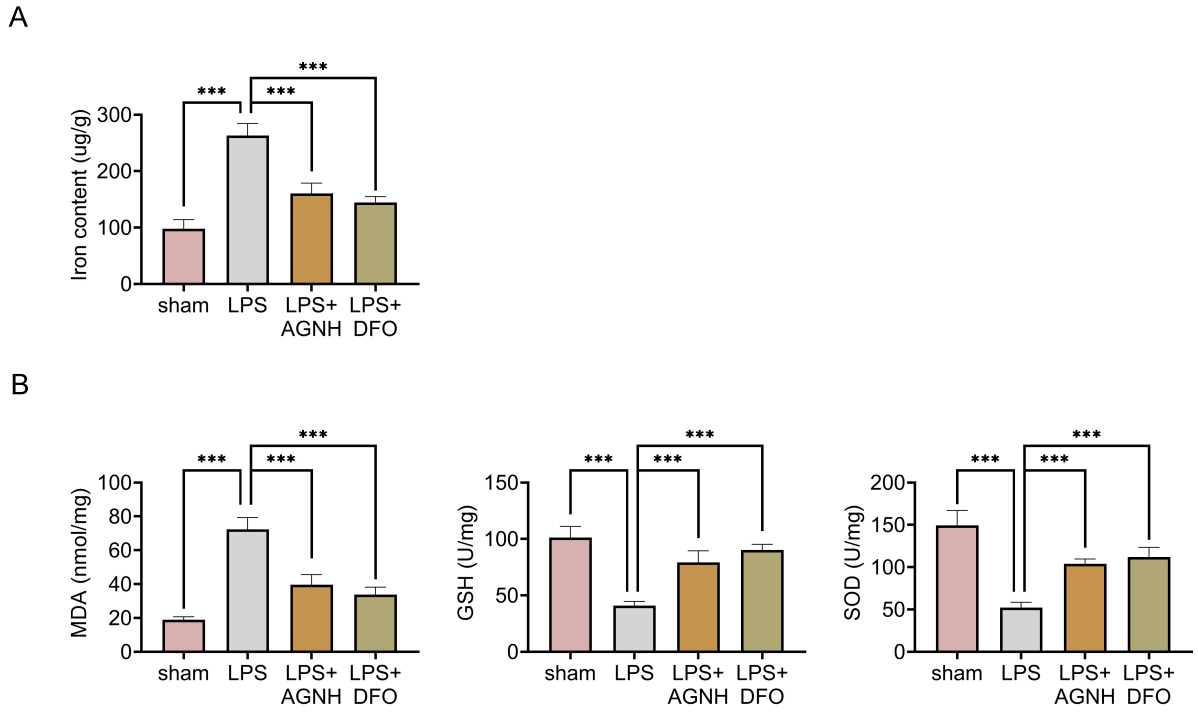

FIGURE 4. AGNH suppressed ferroptosis and oxidative stress in juvenile SAE mice. Mice were separated into the sham, LPS, LPS + 100 mg/kg AGNH and LPS + 100 mg/kg DFO groups. (A) The iron content was tested through the commercial kit. (B) The levels of MDA, GSH and SOD were measured through the corresponding commercial kits. N = 6 mice in each group. ***$p < 0.001$. LPS: lipopolysaccharide; AGNH: Angong Niuhuang Pill; DFO: Defetoxamine; MDA: malondialdehyde; GSH: glutathione; SOD: superoxide dismutase.

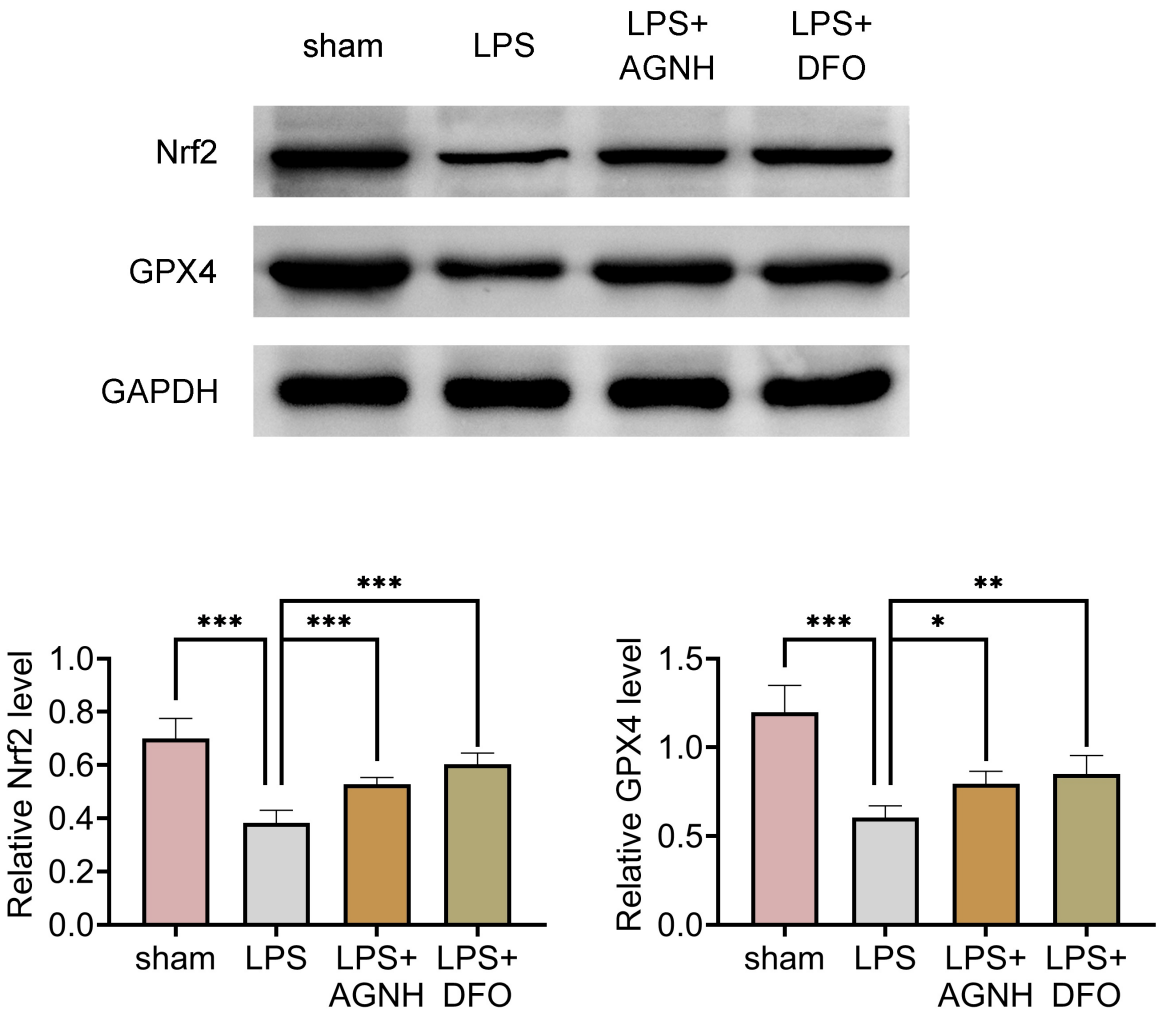

FIGURE 5. AGNH promoted Nrf2/GPX4 signaling pathway. Mice were separated into the sham, LPS, LPS + 100 mg/kg AGNH and LPS + 100 mg/kg DFO groups. The protein expressions of Nrf2 and GPX4 were determined through western blot. N = 6 mice in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. LPS: lipopolysaccharide; AGNH: Angong Niuhuang Pill; DFO: Defetoxamine; Nrf2: nuclear factor erythroid 2-related factor 2; GPX4: glutathione peroxidase 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

such as the lack of human samples, cell models and exploration of other cellular processes. These limitations reduce the generalizability and reliability of this study findings, and hint that the truly employment of AGNH in SAE clinical treatment needs further investigations on cell and human experiments. Future research will encompass additional experiments to investigate further the various roles of AGNH in the progression of SAE.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

AK—Conceptualization, Methodology and Writing-Original Draft; ZHM—Formal analysis, Resources and Investigation; ZA—Formal analysis, Visualization and Data Curation; GA—Project administration, Supervision and Validation; NH, AA—Validation, Supervision and Writing-Review & Editing. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of First Affiliated Hospital of Xinjiang Medical University (Approval No. 20230901-11).

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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