Article

Discovery Medicine 2024; 36(186): 1453–1463
https://doi.org/10.24976/Discov.Med.202436186.135

Levosimendan Ameliorates Hypoxia-Induced Brain Injury in Rats by Modulating PTEN/Akt Signaling Pathway-Mediated Ferroptosis

Wei Meng[1], Chang Shu[2], Hong Gao[1,*]

[1]Department of Anesthesiology, The Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Nanjing Medical University, 214023 Wuxi, Jiangsu, China
[2]Department of Emergency, The Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Nanjing Medical University, 214023 Wuxi, Jiangsu, China
*Correspondence: gh200210180221@126.com (Hong Gao)
Published: 20 July 2024

Background: Levosimendan (Levo) is a drug commonly used to treat heart failure. Recent studies have suggested that Levo may have neuroprotective effects, but it is still unknown how exactly it contributes to hypoxia-induced brain damage. Thus, the aim of this study was to investigate how Levo affects hypoxia-induced brain damage and to clarify any possible underlying mechanisms.
Methods: One group of rats (Levo group) was pretreated with Levo via oral force-feeding for four weeks. Another group (Ferrostatin-1 (Fer-1) group) was pretreated with intraperitoneal injections of Fer-1 for four weeks. A rat model of chronic hypoxia was created by treating rats with 13% O_2 for 14 days in a closed hypoxia chamber. For each group (Control, Model, Levo, Fer-1), we evaluated learning and memory capacity and the morphology and structure of neurons in the rats' brain tissue. Other measurements included tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6); malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px); Fe^{2+}; apoptosis; cleaved caspase-3, caspase-3; phosphatase and tensin homolog (PTEN), protein kinase B (Akt), phosphorylated Akt (p-Akt); and ferroptosis-related proteins Nuclear factor erythroid 2-related factor 2 (Nrf2), glutathione peroxidase 4 (GPX4), and solute carrier family 7 member 11 (SLC7A11).
Results: The Model group rats had considerably fewer neurons than the Control group, with loosely arranged cells, and markedly impaired learning and memory abilities ($p < 0.05$). Oxidative damage and inflammation in brain tissues of the Model group were significantly intensified, accompanied by a substantial increase in neuronal apoptosis ($p < 0.05$). PTEN protein, Fe^{2+} concentration, and cleaved caspase-3 expression were all significantly upregulated, whereas p-Akt, Nrf2, GPX4, and SLC7A11 proteins were dramatically downregulated ($p < 0.05$). Both the Levo and Fer-1 groups demonstrated significantly more neurons and closely arranged cells than the Model group, along with a notable improvement in learning and memory abilities ($p < 0.05$). Oxidative damage and inflammation in brain tissues of the Levo and Fer-1 groups were markedly alleviated, and neuronal apoptosis was suppressed ($p < 0.05$). p-Akt, Nrf2, GPX4, and SLC7A11 proteins were dramatically upregulated, whereas the expression of cleaved caspase-3, PTEN protein, and Fe^{2+} content was considerably downregulated ($p < 0.05$).
Conclusions: Levo effectively mitigates brain injury in rats with chronic hypoxia, likely by regulating ferroptosis via the PTEN/Akt signaling pathway.

Keywords: Levosimendan; chronic hypoxia; PTEN/Akt signaling pathway; ferroptosis; brain injury

Introduction

Chronic hypoxia is a state in which the body is exposed to a low-oxygen environment for a prolonged period of time or has an inadequate supply of oxygen as a result of certain diseases. This state may trigger problems such as cognitive decline, anemia, and myocardial ischemia. The mechanisms of chronic hypoxia involve various aspects of oxygen delivery, oxygen utilization, and tissue demand for oxygen [1]. Chronic hypoxia is a fairly common physiological state in life, encompassing both environmental and tissue hypoxia. People may be at risk for permanent cognitive or memory impairment in situations of environmental hypoxia, such as exposure to high altitudes. This suggests that environmental hypoxia may affect the normal functioning of the central nervous system to some extent. With advancing age, there is a gradual decline in the brain's capacity to utilize oxygen, contributing to the potential onset of diverse neurological disorders, including neurodegenerative diseases [2]. Clinically, hypoxia is a common initiator of histopathological changes due to a variety of diseases, and an important contributor to neurological dysfunction. Brain tissue is the most sensitive to hypoxia. Short periods of hy-

poxia can lead to severe neuronal dysfunction, whereas relatively prolonged hypoxia may eventually lead to cell death [3]. Sustained chronic hypoxia often leads to irreversible brain damage, so it is important to develop new drugs to ameliorate brain damage caused by hypoxia.

Ferroptosis, a novel cell death pathway associated with abnormal cellular iron metabolism, has attracted the attention of scientists in recent years. This type of cell demise is correlated with disrupted intracellular iron levels and the excessive buildup of iron ions. In ferroptosis, the excessive accumulation of intracellular iron ions induces heightened oxidative stress, initiating a sequence of pathological alterations, including the disruption of cell membranes, compromised mitochondrial function, and fragmentation of nuclear DNA [4]. The specific effects of ferroptosis on cell membrane disruption and oxidative stress make it a unique form of cell death compared to other modes of death. Several studies have suggested that hypoxic conditions may lead to intracellular iron accumulation and participate to some extent in hypoxia-induced cellular damage. Iron may trigger cell death by generating free radicals and oxidative stress [5,6]. According to a recent study, N-acetyl serotonin can prevent neuronal ferroptosis in the hippocampal regions following hypoxic brain injury, hence exerting neuroprotective effects [7]. These findings imply that ferroptosis is a major factor in hypoxic brain damage.

Levosimendan (Levo) is an ascending calcium sensitivity modulator with potent antioxidant and anti-inflammatory activity. Frequently employed in heart failure therapy, Levo is known for its ability to enhance heart contractility and dilate blood vessels, thereby improving cardiac pumping efficiency. Numerous preclinical and clinical studies have demonstrated the pleiotropic effects of Levo on various organs [8]. One study showed that Levo could exert a protective effect against endothelial cell death by attenuating inflammatory and oxidative stress pathways [9]. Several studies in recent years have also shown that Levo has neuroprotective effects. Levo is a medication with the ability to easily traverse the blood–brain barrier, exerting central effects. It has demonstrated an enhancement in cerebral blood flow and neuroprotective properties [10]. Protein kinase B (Akt) acts as a pivotal signaling hub governing cell growth, proliferation, and survival [11]. Upstream phosphatase and tensin homolog (PTEN), which reduce Akt phosphorylation and obstruct downstream signaling processes under Akt regulation, affect Akt activation [12]. A previous study documented the alleviating effects of Levo on doxorubicin-induced myocardial injury and the enhancement of cardiac function. These protective actions were attributed to the modulation of the PTEN/Akt signaling pathway, resulting in a reduction in apoptosis [13]. Additionally, another study suggested that Levo treatment could counteract diabetes-induced impairments in learning and memory by preventing changes in the antioxidant system within the hippocampus [14]. Furthermore, an additional study indicated that Levo improved both short- and long-term memory by reinforcing antioxidant defense mechanisms in the hippocampus [15]. A recent study demonstrated that Levo was able to reverse mitochondrial dysfunction, reduce reactive oxygen species (ROS) levels, and inhibit ferroptosis in the myocardial tissues in heart failure with preserved ejection fraction (HFpEF) mice, as evidenced by a reduction in intracellular levels of ferrous ions, malondialdehyde (MDA), and 4-Hydroxynonenal (4-HNE), as well as an increase in the expression of glutathione peroxidase 4 (GPX4), Cystine/glutamate transporter (xCT), and Fibroblast-specific protein 1 (FSP-1) [16]. Based on this, in the present experiment, we took chronic hypoxia model rats as the research object, and explored the mechanism of Levo in improving hypoxic brain injury by observing the behavioral as well as histopathological changes in rats and detecting the levels of inflammatory factors, oxidative indexes, as well as the Fe^{2+} content and ferroptosis-related protein expression, to provide an experimental basis for Levo in clinical treatment.

Materials and Methods

Reagents and Instruments

Reagents: Levosimendan (L5545, Merck, Shanghai, China); RIPA lysing solution, Nissl staining solution (P0013B, C0117, Beyotime, Shanghai, China); hematoxylin and eosin (H&E) staining kit, Bicinchoninic Acid (BCA) Protein Assay kit (C0105S, P0012S, Beyotime, Shanghai, China); Fe^{2+} content detection kit (BC5415, Solarbio, Beijing, China); TdT-mediated dUTP-biotin nick end labeling (TUNEL) kit (C1091, Beyotime, Shanghai, China); interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) Enzyme linked immunosorbent assay (ELISA) kit (SEKR-0005, SEKR-0009, Solarbio, Beijing, China); interleukin-1 beta (IL-1β) ELISA kit (PI303, Beyotime, Shanghai, China); malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) ELISA kit (ml077384, ml059387, ml000618, mlbio, Shanghai, China).

Instruments and equipment: Enzyme Labeling Instrument (A51119700DPC, Thermo Fisher, Waltham, MA, USA); Electrophoresis Instrument (PSC120M, Thermo Fisher, Waltham, MA, USA); Optical microscope (HS-CX40M, hongsheng17, Guangdong, China); Chemiluminescence Instrument (2805621, Thermo Fisher, Waltham, MA, USA); Thermo Centrifuge (46962, Thermo Fisher, Waltham, MA, USA); Thermo Slicer (149AUTO00C1, leicabiosystems, Shanghai, China).

Animal Grouping and Drug Administration

All experimental procedures were approved by the Animal Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University (WY767359).

Twelve 3-month-old male SD rats with a body mass of 180–200 g were obtained from Hunan Slake Jinda Laboratory Animal Co. (hnslkjd001, hnsja, Hunan, China). The rats were randomly divided into Control, Model, Levo, and Ferrostatin-1 (Fer-1, an inhibitor of ferroptosis) groups, with five rats in each group, according to the SPSS random number generation method. Rats in the Fer-1 group received intraperitoneal injections of Fer-1 (HY-100579, MedChemExpress, South Plainfield, NJ, USA, 2 mg/kg) or an equivalent volume of saline once daily for 4 weeks [17]. Concurrently, rats in the Levo group received Levo (1 mg/kg) or an equivalent volume of saline administered once daily via oral force-feeding over the same 4-week period. The solubility and stability of Levo was confirmed prior to the study. Levo doses were selected based on pharmacokinetic and pharmacodynamic data described by Louhelainen *et al*. (2007) [18].

Hypoxia Treatment

The chronic hypoxia rat model was constructed by referring to the methods previously reported in the literature [2]. After the final administration, normal control rats were housed in the animal experimental facility, while the remaining rats in each group underwent hypoxia treatment in a closed hypoxia chamber (China Innovative Instruments Co., Ltd., Ningbo, Zhejiang, China) with the precisely desired hypoxia concentration and pattern. For the chronic hypoxia regimen, rats received continuous treatment with 13% O_2 for a period of 14 days. Every three days, the hypoxic chamber was briefly opened for the addition of food and water. Behavioral tests were conducted at the conclusion of the experimental period. Subsequently, rats were given an intraperitoneal injection of pentobarbital (150 mg/kg) and euthanized by cervical dislocation, and their brain tissues were rapidly dissected on ice and stored at –80 °C.

Behavioral Experiments

Novel Object Recognition Test (NOR)

The left and right edges of a square open field were occupied by two identical objects, designated A1 and A2. The mouse was positioned equally between the two objects, and during the course of the following five minutes, the exploration times (TA1, TA2) for each object were noted. An hour later, a new object, B, took the place of A2; and over the course of the next five minutes, the exploration times of both objects were noted. After twenty-four hours, object B was replaced with a brand-new, completely unrelated object called C, and the process was repeated.

Morris Water Maze (MWM)

At the end of drug administration, the MWM, which involves assessment of localization, navigation, and spatial exploration, was conducted. The number of instances when rats crossed the platform, along with their swimming trajectory and the time spent in different quadrants, was recorded in 60 s to assess the rats' capacity for memory and learning.

Hematoxylin and Eosin Staining (H&E Staining)

The rat brain tissue samples were dewaxed and hydrated, then the samples were immersed in hematoxylin for 5 min, and the residual liquid was rinsed off with tap water. The samples were then differentiated by hydrochloric acid and alcohol for 30 s. Next, the samples were treated with eosin for 30 s, and the residual liquid was rinsed off with tap water, and then the specimens were dried naturally and sealed with neutral gum, and the stained specimens were stored at room temperature and photographed under the light microscope (HS-CX40M, hongsheng17, Guangdong, China) for observation.

Nissl Staining

Brain tissues were removed for paraffin embedding and sectioning, and the sections were deparaffinized to water using xylene and gradient ethanol, rinsed in distilled water, and stained with Nissl staining solution for 10 min, dehydrated and clarified again using gradient ethanol and xylene, and sealed with neutral gum. The sections were observed under a microscope (HS-CX40M, hongsheng17, Guangdong, China).

TUNEL Staining

The fixed brain tissues underwent cryosectioning, and the resulting sections were sequentially immersed in xylene, anhydrous ethanol, and ethanol with varying concentration gradients for 5 min each, followed by three washes with PBS. Subsequently, 50 µL of proteinase K solution (ST533-1mL, Beyotime, Shanghai, China) was added, and the sections were incubated at 37 °C for 30 min, followed by three PBS rinses. Then, 50 µL of trisodium citrate was added dropwise, and the sections were incubated at room temperature for 4 min, followed by three PBS rinses. Staining was performed following the TUNEL kit's (C1091, Beyotime, Shanghai, China) staining steps: the sections were dropwise exposed to 50 µL of TUNEL reaction solution and incubated at 37 °C in the dark for 60 min, followed by three PBS rinses. Next, a peroxidase (POD) conversion solution was added, and the sections were incubated at 37 °C for an additional 30 min. After three PBS rinses, color development was achieved using a diaminobenzidine (DAB) coloring kit (P0202, Beyotime, Shanghai, China), followed by three PBS rinses. The sections were placed in hematoxylin solution for 60 s, the excess staining solution was rinsed out with running water, and then they were immersed in a 1% hydrochloric acid-alcohol solution for differentiation. Following differentiation, the sections were rinsed with running water once more before sealing the film. At the end of the process, the slices were observed and photographed us-

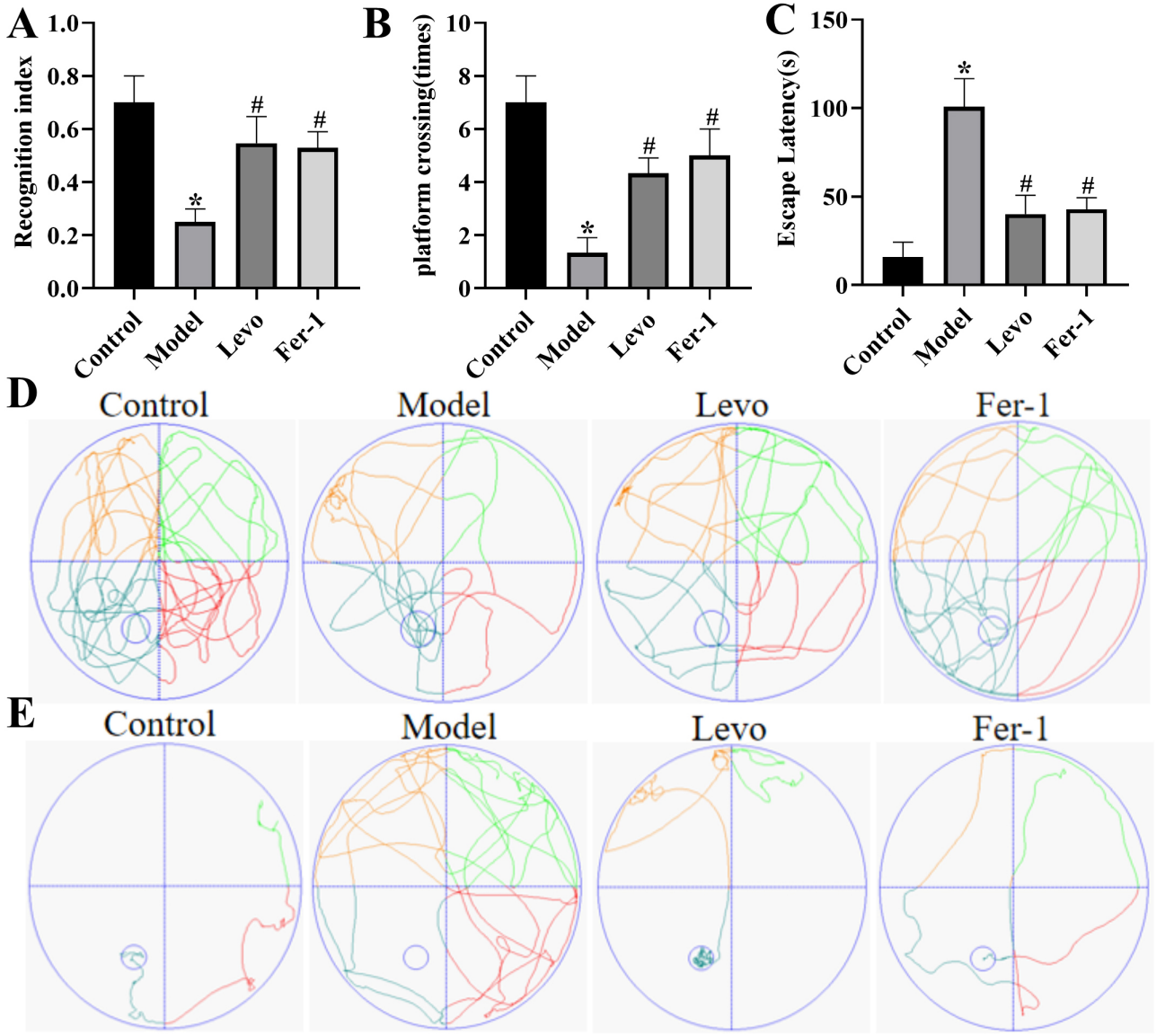

Fig. 1. Effect of Levosimendan (Levo) on chronic hypoxia-induced behavior in rats. (A) The Novel Object Recognition Test. (B–E) Morris water maze. (B,D) Number of platform crossings. (C,E) Platform latency. *$p < 0.05$ vs Control, #$p < 0.05$ vs Model. n = 5. Fer-1, Ferrostatin-1.

ing a CKX53 inverted fluorescence microscope (BX60MF-3M2000F, Olympus, Tokyo, Japan). Statistical analysis was performed using ImageJ (V1.8.0.112, NIH, Madison, WI, USA) image processing software for statistical analysis.

Detection of Fe^{2+} Content

Brain tissue homogenate was utilized for the addition of the Fe^{2+} detection reagent, following the specific procedures outlined in the Fe^{2+} content detection kit's (BC5415, Solarbio, Beijing, China) instructions. Subsequently, the absorbance values were measured at 550 nm using an enzyme labeling instrument, and the Fe^{2+} content was measured from a standard curve.

Western Blotting (WB)

The appropriate amount of total protein extracted from hippocampal tissue was taken for determination, and denatured using 2 × Loading Buffer; 25 µg of total protein sampled in each well underwent SDS-PAGE electrophoresis, and was subsequently transferred to the PVDF membrane (FFP36, Beyotime, Shanghai, China); closed and placed with adding primary antibody in 4 °C for overnight incubation. The next day, the membrane was washed and the secondary antibodies horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG(H+L) (A0208, 1:1000, Beyotime, Shanghai, China) were applied, another round of PBS rinsing ensued, and then enhanced chemiluminescence (ECL) luminescent liquid (P0018AS, Beyotime, Shanghai, China)

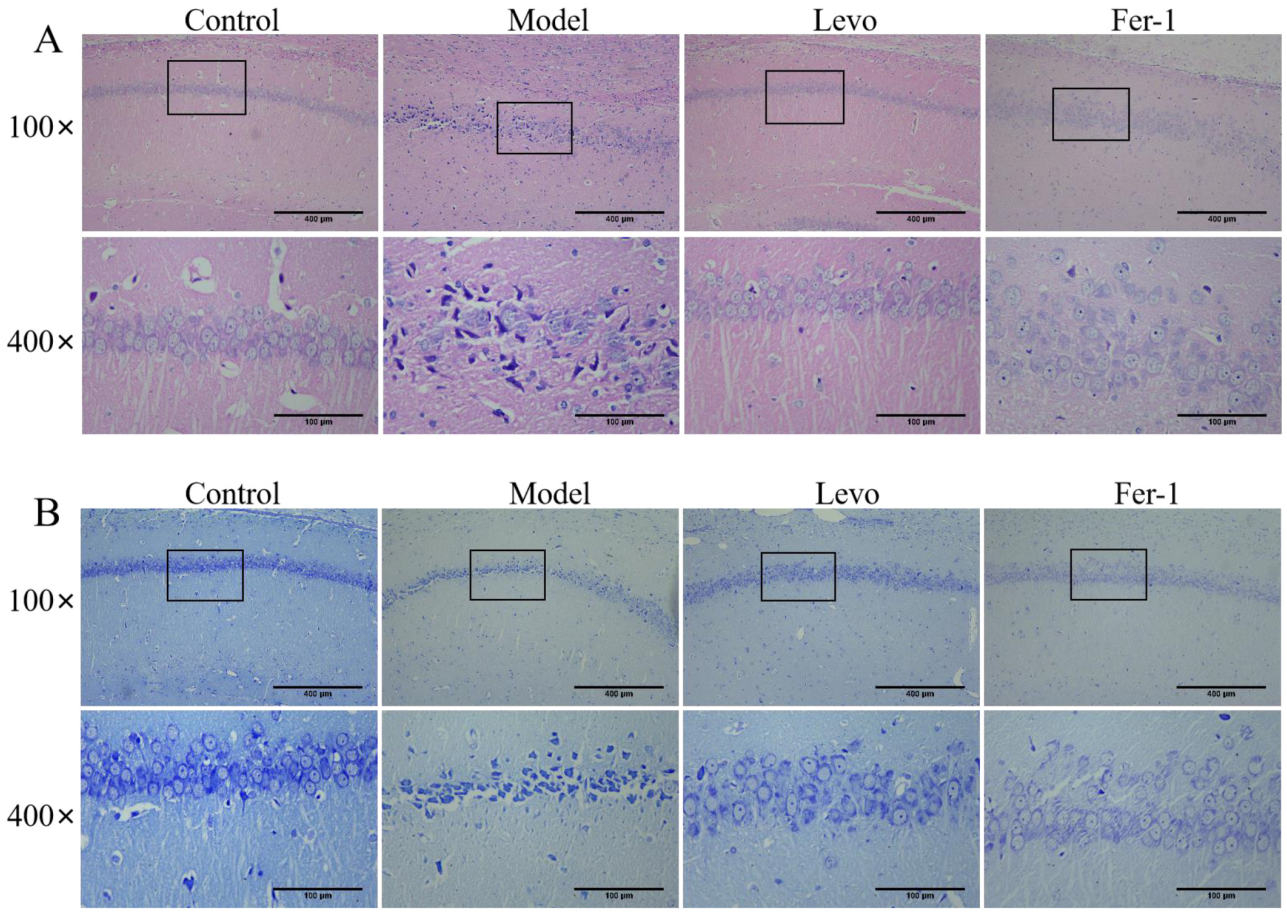

Fig. 2. Effect of Levo on the histopathological morphology of rat brain tissue induced by chronic hypoxia. (A) H&E staining (100×, 400 µm; 400×, 100 µm). (B) Nissl staining (100×, 400 µm; 400×, 100 µm). n = 5. H&E, hematoxylin and eosin; Fer-1, Ferrostatin-1.

was added dropwise for development. The ImageJ software (V1.8.0.112, NIH, Madison, WI, USA) was used to analyze the grayscale values statistically. The primary antibodies used in the experiments were as follows: anti-caspase-3 antibody (A25309, 1:1000, ABclonal, Wuhan, China), anti-cleaved caspase-3 antibody (ab32042, 1:1000, abcam, Shanghai, China), anti-Nuclear factor erythroid 2-related factor 2 (Nrf2) antibody (ab62352, 1:1000, abcam, Shanghai, China), anti-glutathione peroxidase 4 (GPX4) antibody (ab125066, 1:1000, abcam, Shanghai, China), anti-solute carrier family 7 member 11 (SLC7A11) antibody (A2413, 1:1000, ABclonal, Wuhan, China), anti-phosphatase and tensin homolog (PTEN) antibody (A11193, 1:1000, ABclonal, Wuhan, China), anti-phosphorylated Akt (p-Akt) antibody (AP1208, 1:1000, ABclonal, Wuhan, China), anti-Akt antibody (ab38449, 1:1000, abcam, Shanghai, China), anti-GAPDH antibody (ab59164, 1:1000, abcam, Shanghai, China).

Enzyme Linked Immunosorbent Assay (ELISA)

Mouse brain tissue was collected and 10% brain tissue homogenate was prepared by adding PBS (9 mL of PBS to 1 g of tissue sample) according to the mass of the tissue taken. The ELISA kit (IL-6, SEKR-0005, Solarbio, Beijing, China; TNF-α, SEKR-0009, Solarbio, Beijing, China; IL-1β, PI303, Beyotime, Shanghai, China; MDA, ml077384, mlbio, Shanghai, China; SOD, ml059387, mlbio, Shanghai, China; GSH-Px, ml000618, mlbio, Shanghai, China) procedure for IL-6, IL-1β, TNF-α, MDA, SOD, and GSH-Px was followed.

Statistical Analysis

Independent sample *T* test or one-way analysis of variance was performed using GraphPad Prism 9 (Dotmatics, Boston, MA, USA) for sample data. For data with significant differences, the sequential Bonferroni test was used for post hoc comparison. Data are expressed as mean $\pm$ standard deviation, and statistical significance was set at $p < 0.05$.

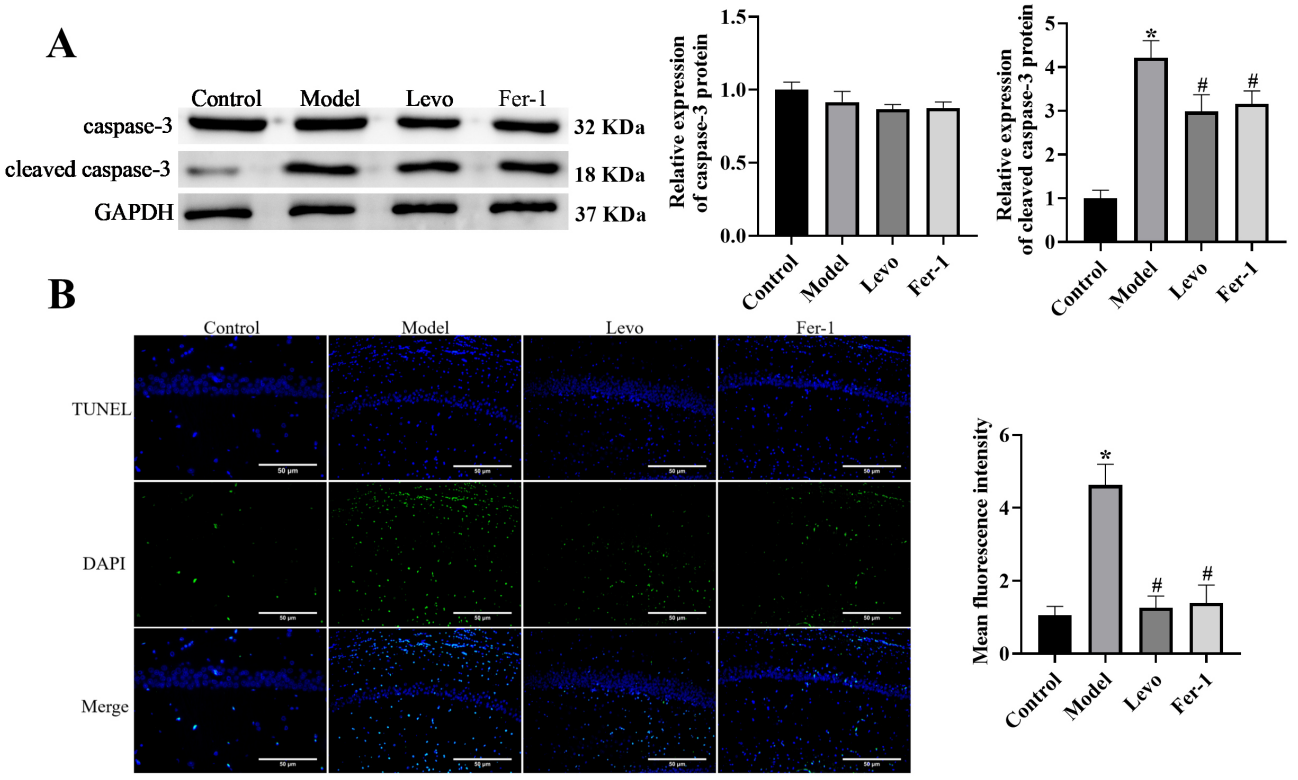

Fig. 3. Effect of Levo on chronic hypoxia-induced apoptosis in rat brain cells. (A) Western Blotting (WB) assay for apoptosis-related protein expression. (B) TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining to detect apoptosis (200×, 50 μm). *$p < 0.05$ vs Control, #$p < 0.05$ vs Model. n = 5.

Results

Effect of Levo on Chronic Hypoxia-Induced Rats' Behavior

Compared to the Control group, the Model group rats showed a markedly lower recognition index (Fig. 1A, $p < 0.05$), increased platform latency and total swimming distance, and a decreased number of platform crossings and time spent in the target quadrant (Fig. 1B–E, $p < 0.05$). When compared to the Model group, Levo and Fer-1 pretreatment reversed these outcomes effectively (Fig. 1, $p < 0.05$). This suggests that Levo may improve the impaired learning and memory abilities induced by chronic hypoxia, possibly through the inhibition of ferroptosis.

Effect of Levo on Chronic Hypoxia-Induced Histopathological Changes in Rat Brain Tissue

According to the results of H&E staining and Nissl staining, the neuronal cells in the Control group had a clear structure, normal morphology, and compact cell arrangement, while rats in the Model group showed a significant reduction in the number of neuronal cells, and the arrangement of cells was sparse. Rats in the Levo and Fer-1 groups, when compared to the Model group, displayed an increased number of neuronal cells and a more compact cell arrangement, as shown in Fig. 2. This suggests that Levo may ameliorate chronic hypoxia-induced brain tissue injury in rats by inhibiting ferroptosis.

Effect of Levo on Chronic Hypoxia-Induced Neuronal Cell Apoptosis in Rats

The apoptosis and cleaved caspase-3 protein expression level of rat neuronal cells in the Model group were significantly higher compared to the Control group (Fig. 3, $p < 0.05$). Conversely, in comparison to the Model group, neuronal cells of both the Levo and Fer-1 groups demonstrated a significant decrease in apoptosis and cleaved caspase-3 protein expression level (Fig. 3, $p < 0.05$). This suggests that Levo inhibits apoptosis in brain cells caused by chronic hypoxia.

Effect of Levo on Oxidative Stress and Inflammatory Cytokines in Rats Induced by Chronic Hypoxia

In comparison to the Control group, the Model group exhibited significantly reduced levels of GSH-Px and SOD in the brain tissues of rats, along with significantly increased levels of MDA, TNF-α, IL-1β, and IL-6 (Fig. 4, $p < 0.05$). Conversely, Levo and Fer-1 pretreatment effectively reversed these alterations when compared to the

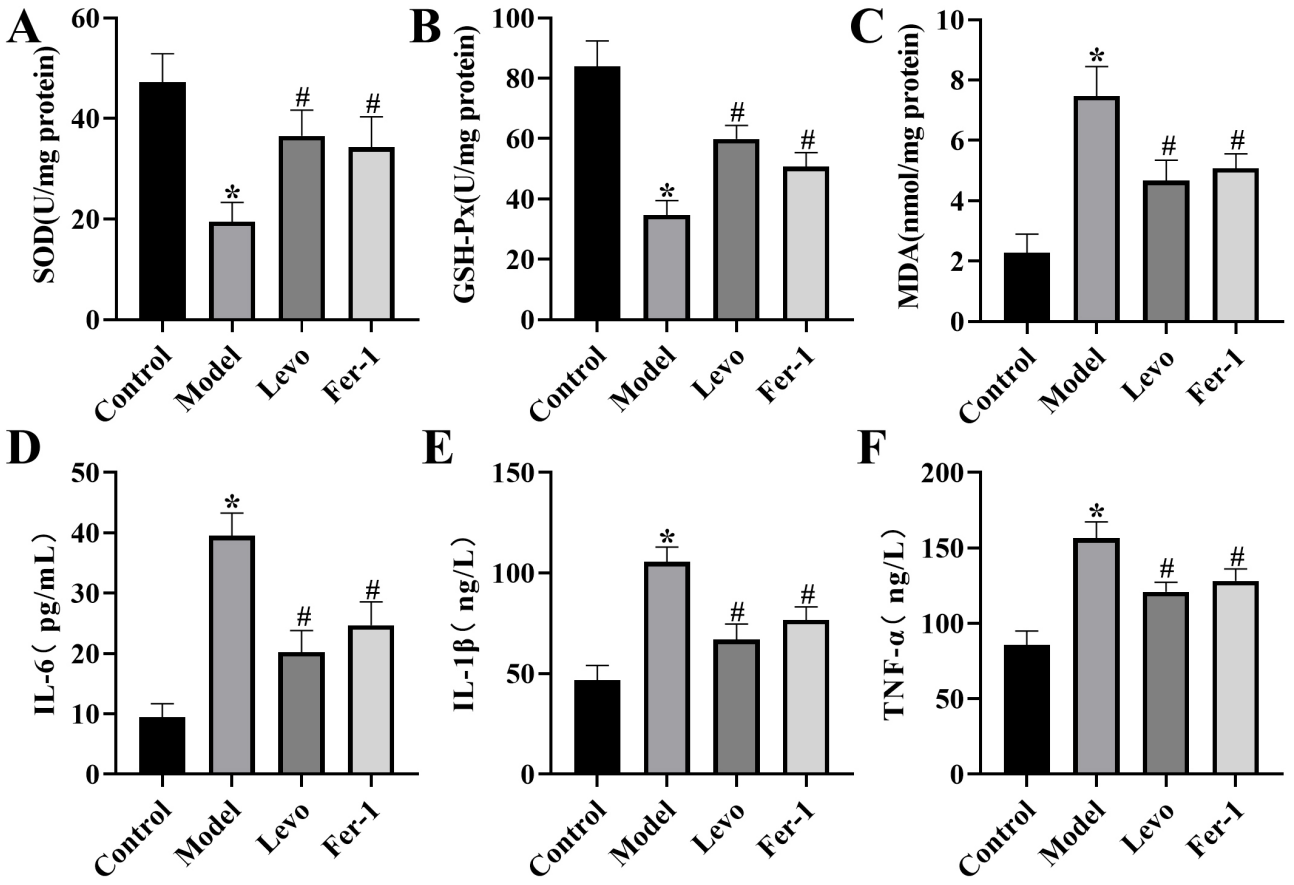

Fig. 4. Effects of Levo on chronic hypoxia-induced oxidative stress and inflammatory cytokines in rats. (A–C) Levels of oxidative indicators detected by Enzyme linked immunosorbent assay (ELISA). (D–F) Levels of inflammatory factors detected by ELISA. *$p < 0.05$ vs Control, #$p < 0.05$ vs Model. n = 5. GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; IL-6, interleukin-6; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor-alpha.

Model group (Fig. 4, $p < 0.05$). This suggests that Levo may inhibit oxidative damage and inflammatory response caused by chronic hypoxia by inhibiting ferroptosis.

Effect of Levo on Fe^{2+} Content and Ferroptosis-Related Protein Expression in Rats Induced by Chronic Hypoxia

In comparison to the Control group, the Model group exhibited considerably higher Fe^{2+} content in the brain tissue, along with significantly lower levels of ferroptosis-related proteins Nrf2, GPX4, and SLC7A11 (Fig. 5, $p < 0.05$). Conversely, Levo and Fer-1 pretreatment effectively reversed these alterations when compared to the Model group (Fig. 5, $p < 0.05$). This implies that Levo can inhibit ferroptosis caused by chronic hypoxia.

Effect of Levo on the Expression of PTEN/Akt Pathway-Related Proteins in Rats Induced by Chronic Hypoxia

In contrast to the Control group, the Model group exhibited a significant increase in PTEN level and a significant decrease in the p-Akt/Akt level in the brain tissue of rats (Fig. 6, $p < 0.05$). However, Levo and Fer-1 pretreatment effectively reversed these changes when compared to the Model group (Fig. 6, $p < 0.05$). This indicates that Levo may inhibit ferroptosis in rats with chronic hypoxia through modulation of the PTEN/Akt pathway.

Discussion

The intracellular milieu of the mammalian brain is highly susceptible to perturbations resulting from external alterations or trauma, and hypoxia in particular can trigger neuronal death within minutes, leading to severe impairment of brain function. Impairment of neuronal function may have serious adverse consequences for the organism. A variety of diseases, such as stroke, cardiovascular system dysfunction, respiratory diseases, and hematologic diseases, may cause chronic hypoxia, which in turn exacerbates brain damage [19]. One study has highlighted the correlation between chronic hypoxia and cognitive dysfunction [20]. Our findings indicate that in comparison to the Control group, rats in the Model group exhibited a considerable decline in learning and memory abilities. Histopathological

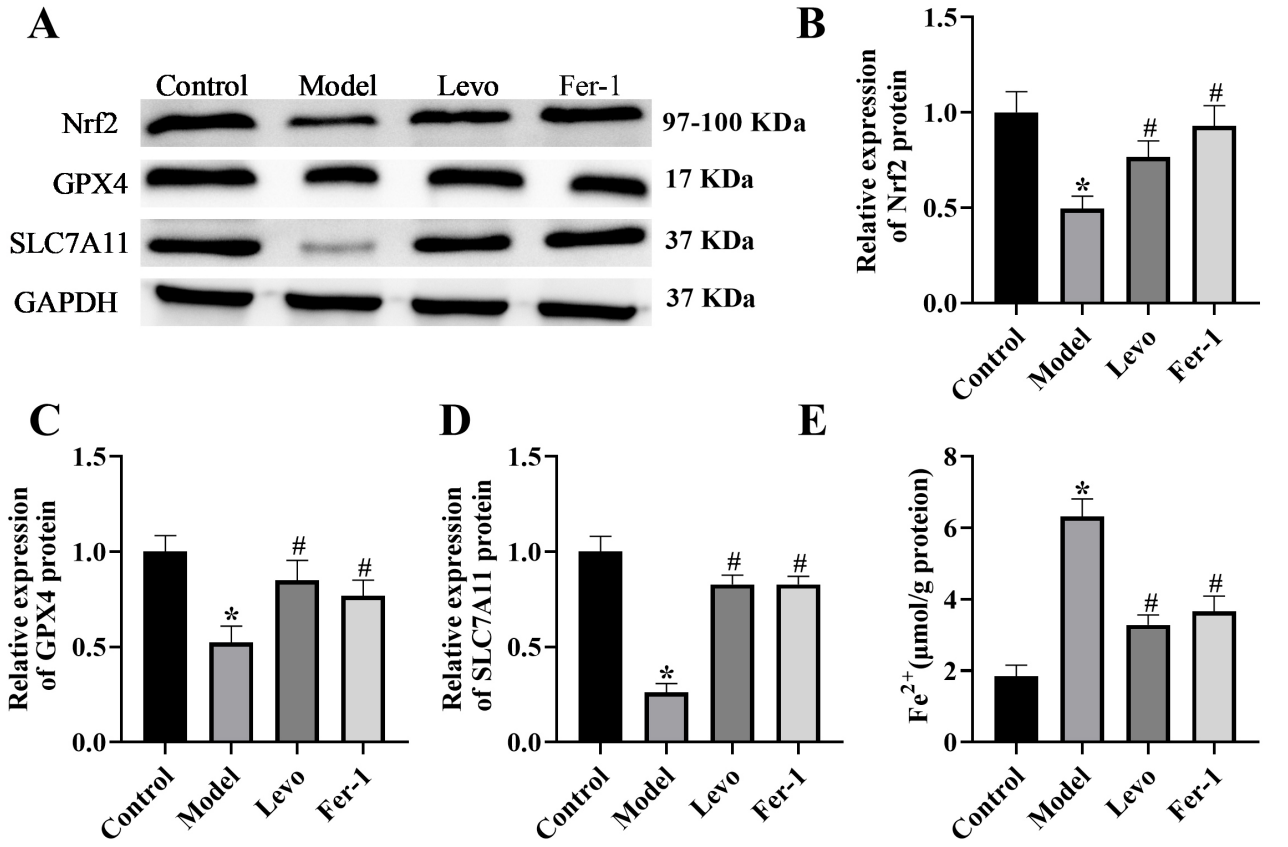

Fig. 5. Effect of Levo on Fe^{2+} content and expression of iron death-related proteins in rats induced by chronic hypoxia. (A–D) Iron death-related protein expression level detected by WB method. (E) Fe^{2+} content assay. *$p < 0.05$ vs Control, #$p < 0.05$ vs Model. n = 5. Nrf2, Nuclear factor erythroid 2-related factor 2; GPX4, glutathione peroxidase 4; SLC7A11, solute carrier family 7 member 11.

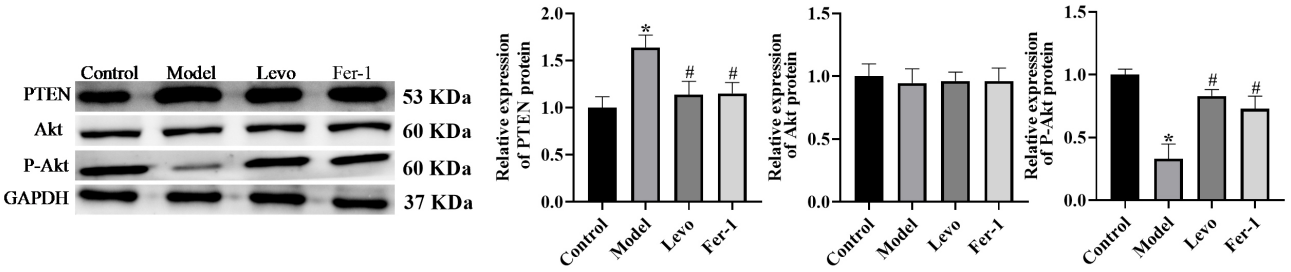

Fig. 6. Effect of Levo on the PTEN/Akt pathway-related protein expression in chronic hypoxia-induced rats. *$p < 0.05$ vs Control, #$p < 0.05$ vs Model. n = 5. PTEN, phosphatase and tensin homolog; Akt, protein kinase B; p-Akt, phosphorylated Akt.

observations revealed a reduction in the number of neuronal cells and sparse cell arrangement in rats from the Model group compared to the Control group. This suggests that we have successfully constructed a model of sustained chronic hypoxia. Another study reported that Levo may exert neuroprotective effects by reducing necrosis in traumatic brain injury [10]. Our experimental results showed that Levo was able to ameliorate hypoxia-induced decline in learning and memory abilities and brain tissue damage. This implies that Levo may exert neuroprotective effects by inhibiting ferroptosis.

Hypoxia-induced cellular damage can activate the immune system and create an inflammatory response. The secreted inflammatory mediators may cause further damage to the surrounding cells. In addition, persistent hypoxic conditions may induce excessive apoptosis, thereby exacerbating brain damage [21]. It has been shown that activated Akt may exert its anti-apoptotic effects by regulating caspase-3 [22]. Caspase-3 serves as a crucial effector in apoptosis, existing in a precursor form under normal conditions and becoming activated during the process of apoptosis. Levo, beyond its cardiac-enhancing effects, encompasses various cardiovascular benefits, including anti-

inflammatory and anti-apoptotic properties. Research indicates that Levo preconditioning provides protection against ischemia-reperfusion-induced cardiac dysfunction by slowing myocardial apoptosis, and this protective mechanism is associated with its regulation of the PTEN/Akt signaling pathway [23]. Additionally, studies suggest that Levo reduces mortality post-cardiac arrest and cardiopulmonary resuscitation, as well as mitigates acute kidney injury by suppressing inflammation and apoptosis while activating the ERK signaling pathway [24,25]. In chronic hypoxic rats, the current study demonstrated that both Levo and Fer-1 pretreatment markedly decreased the rate of neuronal apoptosis, levels of cleaved caspase-3 protein expression, and inflammatory factor levels. This suggests that Levo may ameliorate apoptosis and inflammatory responses caused by chronic hypoxia by inhibiting ferroptosis.

Cellular ferroptosis is intricately associated with the disruption of intracellular iron ion metabolism and oxidative damage. When cellular ferroptosis occurs, Fe^{2+} accumulates in the cell, and due to its strong oxidative properties, Fe^{2+} can react with intracellular H_2O_2, causing a large number of hydroxyl radicals to be generated, which promotes the occurrence of lipid peroxidation and damages cellular membranes [26]. One of the main natural defense systems of the body against oxidative stress and neuroinflammation is Nrf2-mediated signaling, which also plays a vital part in oxidative stress [27]. Nrf2 is regulated by Akt, which is an important transcription factor for maintaining cellular redox homeostasis. GPX4 is a key protein regulating cellular ferroptosis, and its reduced expression disrupts intracellular oxidative homeostasis and induces the onset of lipid peroxidation, thus promoting cellular ferroptosis [28]. Activation of the Akt-Nrf2 pathway can lead to elevated expression of downstream GPX4 and inhibit the occurrence of cellular ferroptosis [29]. SLC7A11, a crucial component of cystine/glutamate transporter proteins, plays a vital role in regulating cellular lipid peroxidation and suppressing iron accumulation [30]. A recent study revealed that hypoxia could induce neurodegeneration in neonatal animals by elevating brain iron levels. Hypoxia affects neuronal cell metabolism through GPX4 and SLC7A11, leading to an excess of oxygen free radicals and reduced SOD activity. This cascade results in the generation of substantial ROS from the lipid peroxidation of cell membrane polyunsaturated fatty acids (PUFAs), ultimately triggering ferroptosis [31]. Another study demonstrated that Levo could enhance cardiac function by activating connexin 43-mediated mitochondrial protection and providing continuous inhibition of ferroptosis in cardiomyocytes within a mouse model of metabolic syndrome [16]. The outcomes of our study revealed that, in chronic hypoxic rats, both Levo and Fer-1 pretreatments significantly elevated GSH-Px and SOD levels in brain tissues while reducing intracellular Fe^{2+} content and MDA levels. Moreover, Levo and Fer-1 pretreatments decreased the expression level of PTEN protein while increasing the expression levels of p-Akt/Akt, GPX4, Nrf2, and SLC7A11 proteins. These findings suggest that Levo can mitigate brain injury by inhibiting hypoxia-induced ferroptosis, potentially through regulation of the PTEN/Akt pathway.

This study reveals the potential role of Levo in alleviating chronic hypoxia-induced brain injury, but some limitations still need to be considered. First, animal models cannot fully mimic human physiology and pathology, so the applicability of the findings in humans remains to be verified, and more clinical data are needed to confirm them. Second, this study lacks an in-depth exploration of molecular mechanisms and cellular pathways and fails to fully analyze the mechanism of Levo's action in brain injury. In addition, this study only investigated the pretreatment effect of Levo, and the assessment of long-term therapeutic effects requires longer observation and evaluation. Future studies need to validate these findings, explore the interactions between Levo and cellular pathways, and conduct longer-term therapeutic observations to fully assess its potential value in the treatment of brain injury. In summary, despite the important findings of this study, there are still some limitations that need to be overcome. Future studies should continue to explore the therapeutic mechanisms of Levo in depth, conduct more clinical trials to verify their feasibility and effectiveness in clinical applications, and take appropriate measures to minimize the limitations and provide better treatment options for patients.

Conclusions

Levo was demonstrated to effectively mitigate brain injury in rats exposed to chronic hypoxia, and its mechanism of action appears to be associated with the regulation of ferroptosis through the PTEN/Akt signaling pathway. This suggests that Levo may serve as a potential therapeutic intervention for diseases associated with chronic hypoxia. However, additional in-depth studies are necessary to clarify its molecular mechanisms and determine clinical feasibility.

Availability of Data and Materials

The data used and/or analyzed during the current study are available from the corresponding author.

Author Contributions

WM and CS designed the research study. CS and HG performed the research. WM provided help and advice on the experiments. HG analyzed the data. WM wrote the first draft. All authors contributed significantly to editorial changes of important content. All authors 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

This study was approved by the Animal Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University (WY767359).

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Lee JW, Ko J, Ju C, Eltzschig HK. Hypoxia signaling in human diseases and therapeutic targets. Experimental & Molecular Medicine. 2019; 51: 1–13.

[2] Li G, Liu J, Guo M, Gu Y, Guan Y, Shao Q, *et al*. Chronic hypoxia leads to cognitive impairment by promoting HIF-2α-mediated ceramide catabolism and alpha-synuclein hyperphosphorylation. Cell Death Discovery. 2022; 8: 473.

[3] Goodall S, Twomey R, Amann M. Acute and chronic hypoxia: implications for cerebral function and exercise tolerance. Fatigue: Biomedicine, Health & Behavior. 2014; 2: 73–92.

[4] Tang D, Chen X, Kang R, Kroemer G. Ferroptosis: molecular mechanisms and health implications. Cell Research. 2021; 31: 107–125.

[5] Gao X, Hu W, Qian D, Bai X, He H, Li L, *et al*. The Mechanisms of Ferroptosis Under Hypoxia. Cellular and Molecular Neurobiology. 2023; 43: 3329–3341.

[6] Zheng X, Liang Y, Zhang C. Ferroptosis Regulated by Hypoxia in Cells. Cells. 2023; 12: 1050.

[7] Yang X, Yang Y, Gao F, Lu K, Wang C. N-Acetyl Serotonin Provides Neuroprotective Effects by Inhibiting Ferroptosis in the Neonatal Rat Hippocampus Following Hypoxic Brain Injury. Molecular Neurobiology. 2023; 60: 6307–6315.

[8] Pan J, Yang YM, Zhu JY, Lu YQ. Multiorgan Drug Action of Levosimendan in Critical Illnesses. BioMed Research International. 2019; 2019: 9731467.

[9] Rodríguez-González R, Pollesello P, Baluja A, Álvarez J. Effects of Levosimendan on Inflammation and Oxidative Stress Pathways in a Lipopolysaccharide-Stimulated Human Endothelial Cell Model. Biological Research for Nursing. 2019; 21: 466–472.

[10] Aycan A, Oksuz E, Gonullu E, Kume T, Ergur B, Akyol ME, *et al*. A New Approach in the Treatment of Traumatic Brain Injury: The Effects of Levosimendan on Necrosis, Apoptosis, and Oxidative Stress [published correction appears in World Neurosurgery. 2023; 173: 281]. World Neurosurgery. 2022; 168: e432–e441.

[11] Bi X, Zhang G, Wang X, Nguyen C, May HI, Li X, *et al*. Endoplasmic Reticulum Chaperone GRP78 Protects Heart From Ischemia/Reperfusion Injury Through Akt Activation. Circulation Research. 2018; 122: 1545–1554.

[12] Jiang Y, Chang H, Chen G. Effects of microRNA-20a on the proliferation, migration and apoptosis of multiple myeloma via the PTEN/PI3K/AKT signaling pathway. Oncology Letters. 2018; 15: 10001–10007.

[13] Li LL, Wei L, Zhang N, Wei WY, Hu C, Deng W, *et al*. Levosimendan Protects against Doxorubicin-Induced Cardiotoxicity by Regulating the PTEN/Akt Pathway. BioMed Research International. 2020; 2020: 8593617.

[14] Rababa'h AM, Alzoubi KH, Atmeh A. Levosimendan enhances memory through antioxidant effect in rat model: behavioral and molecular study. Behavioural Pharmacology. 2018; 29: 344–350.

[15] Rababa'h AM, Alzoubi KH, Baydoun S, Khabour OF. Levosimendan Prevents Memory Impairment Induced by Diabetes in Rats: Role of Oxidative Stress. Current Alzheimer Research. 2019; 16: 1300–1308.

[16] Zhang LL, Chen GH, Tang RJ, Xiong YY, Pan Q, Jiang WY, *et al*. Levosimendan Reverses Cardiac Malfunction and Cardiomyocyte Ferroptosis During Heart Failure with Preserved Ejection Fraction via Connexin 43 Signaling Activation. Cardiovascular Drugs and Therapy. 2023. (online ahead of print)

[17] Liu Y, Zhao Z, Guo J, Ma Y, Li J, Ji H, *et al*. Anacardic acid improves neurological deficits in traumatic brain injury by anti-ferroptosis and anti-inflammation. Experimental Neurology. 2023; 370: 114568.

[18] Louhelainen M, Vahtola E, Kaheinen P, Leskinen H, Merasto S, Kytö V, *et al*. Effects of levosimendan on cardiac remodeling and cardiomyocyte apoptosis in hypertensive Dahl/Rapp rats. British Journal of Pharmacology. 2007; 150: 851–861.

[19] Yager JY, Wright S, Armstrong EA, Jahraus CM, Saucier DM. The influence of aging on recovery following ischemic brain damage. Behavioural Brain Research. 2006; 173: 171–180.

[20] Dodd JW, Charlton RA, van den Broek MD, Jones PW. Cognitive dysfunction in patients hospitalized with acute exacerbation of COPD. Chest. 2013; 144: 119–127.

[21] Luo Z, Tian M, Yang G, Tan Q, Chen Y, Li G, *et al*. Hypoxia signaling in human health and diseases: implications and prospects for therapeutics. Signal Transduction and Targeted Therapy. 2022; 7: 218.

[22] Xiao L, Yin XC, Cao QQ. The role of the PI3K/AKT signaling pathway in DADS-induced apoptosis of K562 cells. Chinese Journal of Contemporary Pediatrics. 2016; 18: 1050–1054. (In Chinese)

[23] Wang J, Chen H, Zhou Y, Su Q, Liu T, Li L. Levosimendan Pretreatment Inhibits Myocardial Apoptosis in Swine after Coronary Microembolization. Cellular Physiology and Biochemistry. 2017; 41: 67–78.

[24] Tian L, Wang S, Zhao L, Lu X, Zhu C, Gong H, *et al*. Renoprotective effects of levosimendan on acute kidney injury following cardiac arrest via anti-inflammation, anti-apoptosis, and ERK activation. FEBS Open Bio. 2021; 11: 2236–2244.

[25] Ma Z, Jin H, Liu F, Wang S, Huang P, Fang X. A Meta-Analysis of the Effects of Levosimendan on Cardiac Function and Outcomes in Patients with Sepsis. Alternative Therapies in Health and Medicine. 2023; 29: 668–673.

[26] Li J, Cao F, Yin HL, Huang ZJ, Lin ZT, Mao N, *et al*. Ferroptosis: past, present and future. Cell Death & Disease. 2020; 11: 88.

[27] Han CW, Kwun MJ, Kim KH, Choi JY, Oh SR, Ahn KS, *et al*. Ethanol extract of Alismatis Rhizoma reduces acute lung inflammation by suppressing NF-κB and activating Nrf2. Journal of Ethnopharmacology. 2013; 146: 402–410.

[28] Wang Y, Yan S, Liu X, Deng F, Wang P, Yang L, *et al*. PRMT4 promotes ferroptosis to aggravate doxorubicin-induced cardiomyopathy via inhibition of the Nrf2/GPX4 pathway. Cell Death and Differentiation. 2022; 29: 1982–1995.

[29] Wei N, Lu T, Yang L, Dong Y, Liu X. Lipoxin A4 protects primary spinal cord neurons from Erastin-induced ferroptosis by activating the Akt/Nrf2/HO-1 signaling pathway. FEBS Open Bio. 2021; 11: 2118–2126.

[30] Zhao X, Gao M, Liang J, Chen Y, Wang Y, Wang Y, *et al*. SLC7A11 Reduces Laser-Induced Choroidal Neovascularization by Inhibiting RPE Ferroptosis and VEGF Production. Frontiers in Cell and Developmental Biology. 2021; 9: 639851.

[31] Chang XW, Zhao AP, Yao WT, Li WB, Wang R. Research progress of ferroptosis in hypoxia-associated brain injury. Acta Physiologica Sinica. 2023; 75: 255–268. (In Chinese)